Interaction of enteric viruses with aquatic biofilms in the urban water cycle

Dissertation

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“*Don’t panic*”

- Douglas Adams -

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Glossary

AB/AM	antibiotic/antimycotic
ATCC	American Type Culture Collection
BF	biofilm
CFU	colony forming units
CPE	cytopathic effect
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonocleic acid
DPBS	Dulbecco’s phosphate buffered saline
EMA	ethidium monoazide
EPDM	ethylene propylene diene monomer
EPS	extracellular polymeric substances
EV	enterovirus
FBS	fetal bovine serum
gen.eq.	genome equivalents
HAdV	human adenovirus
HDPE	high-density polyethylene
HEV	hepatitis E virus
HTLV-1	T-cell leukemia virus type 1
HPC	heterotrophic plate count
IEP	isoelectric point
LB	lysogeny broth

MNV	murine norovirus
MPN	most probable number
MSA	Modified Scholtens' Agar
MSB	Modified Scholtens' Broth
NoV	norovirus
PAdV	porcine adenovirus
PBS	phosphate buffered saline
PEG	polyethylene glycol
PFU	plaque forming units
PMA	propidium monoazide
PPMCC	Pearson product-moment correlation coefficient
PVC	polyvinyl chloride
PYG	peptone yeast glucose
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RoV	rotavirus
rpm	revolutions per minute
SD	standard deviation
SP	sampling point
ssMSA	semi-solid Modified Scholtens' Agar
$TCID_{50}$	50% tissue culture infective dose
uPVC	unplasticized polyvinyl chloride
v/v	volume per volume
VIRADEL	virus adsorption elution
w/v	weight per volume

Abstract

Aquatic biofilms are ubiquitous in natural and technical systems of the urban water cycle, where they can act as reservoirs for hygienically relevant microorganisms. Numerous studies have investigated the retention or multiplication of bacteria in different types of biofilms. However, little is known about the interaction of viruses with aquatic biofilms. The aim of this study was to determine the potential of aquatic biofilms in the urban water cycle to retain infectious viruses, including human enteric viruses, animal viruses and bacteriophages. Two field studies were conducted to determine the occurrence of viruses in biofilms from natural and technical systems in the urban water cycle. Moreover, laboratory experiments were performed to study the accumulation of selected viruses in drinking water biofilms and to elucidate the relevance of biological interactions between viruses and biofilms.

In a field study involving the urban River Ruhr in Germany, the distribution of human enteric viruses, bacterial and viral indicator organisms between water, biofilms and sediments was assessed. First, different protocols for the isolation of viruses from biofilms and sediments were compared in recovery experiments, a method based on ultrasonication in elution buffer in combination with a subsequent concentration step was most efficient to isolate human adenovirus (HAdV) and coliphage φX174. During this field study, concentrations of bacterial and viral indicator organisms (*E. coli* and somatic coliphages) were elevated in biofilms and sediments compared to the bulk water by 3-4 log-units, using cultural methods. A period of enhanced rainfall led to a significant increase of *E. coli* and somatic coliphage concentrations in water, whereas concentrations in biofilms and sediments remained relatively constant. Water, biofilms and sediments were also analyzed for the occurrence of selected enteric viruses including human adenovirus (HAdV), enterovirus (EV), group A rotavirus (RoV) and norovirus genogroup GII (NoV GII). Although the detection frequency for enteric viruses in water was higher, HAdV and EV were detected in biofilms and sediments, using quantitative real-time PCR (qPCR).

In a second field study, water and biofilms from drinking water systems for animals from piglet breeding farms in Germany were analyzed for the occurrence of somatic coliphages and selected animal pathogenic viruses. This study revealed a high prevalence of somatic coliphages in biofilms compared to the bulk water, also porcine adenoviruses (PAdV) were frequently detected in water and biofilms, using qPCR. Besides, hepatitis E viruses (HEV) were found in water and biofilms during this field study, however these results are doubtful

because the detection via qPCR was shown to be unspecific. Further research is needed to verify the occurrence of HEV in drinking water systems for animals.

In laboratory experiments, artificial drinking water biofilms were grown on ethylene propylene diene monomer (EPDM) coupons and incubated with three viruses (human adenovirus (HAdV), murine norovirus (MNV) and coliphage φX174) under different hydraulic and physicochemical conditions in order to simulate various situations in a drinking water distribution system. Viruses were quantified in water and biofilms at different time points using qPCR and cultural methods (viral cell culture, plaque assay) in order to determine the distribution between water and biofilms. This work showed that the concentration of all three viruses in water decreased significantly over time, while viruses were enriched in drinking water biofilms by a factor of up to 3.5 log units compared to the water phase within 24 h of incubation. Increased accumulation of viruses in biofilms was observed under stagnant conditions compared to flow conditions. Using cultural methods for virus quantification, the highest accumulation was found for HAdV in artificially hardened water (containing 4 mM Ca^{2+}) under stagnant conditions, indicating a potential influence of the water hardness on virus retention in biofilms. Moreover, the murine macrophage cell line RAW 264.7 showed cytopathic effects when exposed to drinking water or drinking water biofilms without viruses and thus was not suitable to assess the concentration of infectious MNV in drinking water and drinking water biofilms.

In the last part of this work, potential biological interactions of viruses with aquatic biofilms were elucidated. First, it was shown that a propagation of somatic coliphage φX174 in the presence of *E. coli* as natural host under simulated environmental conditions within 4 h at 20 °C or 36 °C is negligible. Moreover, *Acanthamoeba castellanii* was found to incorporate human adenoviruses in co-culture within 7 days of incubation, indicating that amoebae could potentially affect the persistence of viruses in aquatic environments. However, further research is needed to determine the relevance of amoebae in the context of aquatic biofilms.

Overall, this work showed that aquatic biofilms in natural and technical systems can act as reservoirs for viral indicators as well as relevant human and animal pathogenic viruses. Aquatic biofilms can retain viruses that are present in the bulk water and potentially release them after a certain time, which underlines the significance of aquatic biofilms in water hygiene. Besides, this work suggests that biological interactions within aquatic biofilms could affect the fate of viruses in aquatic ecosystems.

1. Introduction

1.1 The urban water cycle

Water is the most important resource for life on earth and is used by humans as drinking water, for production of food, irrigation, bathing, industry and many other purposes. On the other hand, water can also favor transmission of several infectious diseases with mild to severe symptoms. Nowadays, increasing world population and urbanization lead to temporary impairment of water quality and quantity, thus measures for water hygiene and sanitation are required to prevent transmission of waterborne infectious diseases (WHO, 2018). In 2010, safe drinking water was attributed as a human right by the United Nations General Assembly (UN, 2010), however water scarcity and pollution still affect a high percentage of the world's population. In 2015, 844 million people still lacked even basic drinking water services, while 2.3 billion people did not have access to basic sanitation facilities (WHO and UNICEF, 2017).

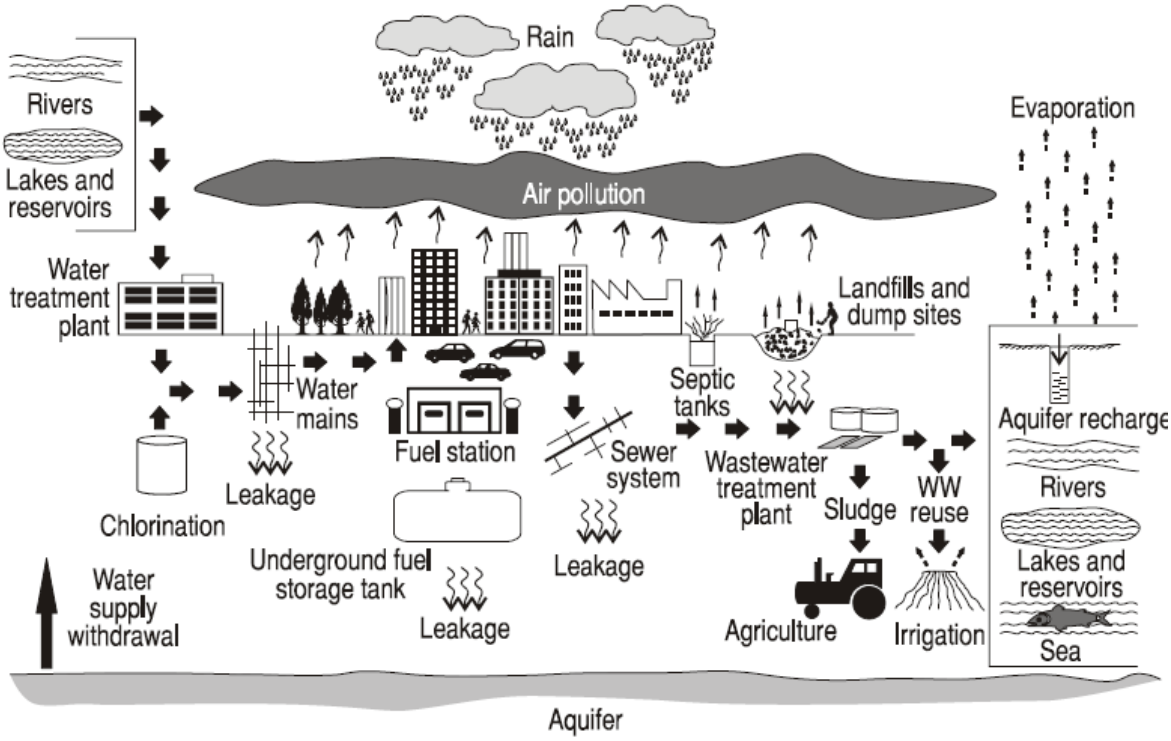

Figure 1: Schematic overview of the urban water cycle. (Marsalek et al., 2006)

The flow of water can be described by the hydrological cycle, which includes the circulation of water between biosphere, atmosphere, lithosphere and hydrosphere (Marsalek et al., 2006). As a part of the hydrological cycle, the urban water cycle (Figure 1) can be used to illustrate the flow of water in urbanized settings, which includes the various influences of human life on the water cycle (e.g. abstraction of raw water for drinking water production, drainage and

wastewater management). After use, water with anthropogenic pollution enters the urban water cycle either as industrial or domestic wastewater, comprising different chemical or microbiological pollutants. Subsequently, wastewater is often mixed with rain water in combined sewer systems, which dilutes pollutants but increases the total amount of wastewater to be treated (Lucas et al., 2014). In some cases, wastewater is separated at the source depending on the level of pollution to reduce efforts for treatment. For instance, grey water is commonly used for purposes such as irrigation after minor treatment (Mohamed et al., 2013; Shi et al., 2018). On the other hand, the major part of the raw sewage is transported to wastewater treatment plants, where it undergoes treatment before it is either used for irrigation, industrial processes, groundwater enrichment (reclaimed water) or released into natural water bodies such as surface waters. Typically, raw water for drinking water production is abstracted from natural waters (surface waters, groundwater) and subsequently purified by different treatment steps. The complexity of the urban water cycle and individual systemic prerequisites concerning geography, infrastructure and population influence the type and extent of contact between humans and water. The limited capacity of natural water resources and insufficient treatment of anthropogenically polluted waters result in the fact that a high percentage of the world's population is exposed to chemically and microbially contaminated waters as described above. Depending on several factors, humans interact with different kinds of water from the urban water cycle, which also determines their personal risk of acquiring waterborne infections.

The relevance of the interplay between pathogens and the aquatic environment as such is not new, but the transmission of pathogens and their impact on human health is complex and includes various disciplines. Consequently, the One Health concept was first discussed in 2004 and was subsequently endorsed by various institutions, including the WHO, the US Centers for Disease Control and Prevention (CDC), the European Commission and many others. According to the One Health concept, human health is connected to the health of animals and the environment (CDC, 2018). This implies that a holistic and interdisciplinary approach on regional and global scales is required to prevent the transmission of infectious diseases. The concept gained increasing attention in recent years due to changes such as globalization, urbanization, environmental pollution or climate change (Destoumieux-Garzon et al., 2018). As major aim, One Health should facilitate the communication and collaboration between researchers from individual disciplines, governments and other institutions in order to improve monitoring and control of diseases. However, removing interdisciplinary barriers

between environmental-, ecological- and evolutionary sciences, human- and animal medicine is still regarded as a major challenge today (Destoumieux-Garzon et al., 2018).

In water hygiene, especially excreta-related pathogens are relevant for human health. These pathogens include bacteria, viruses, protozoa and helminths (WHO, 2018) and can be transmitted via ingestion, inhalation, aspiration or direct contact. Excreta-related pathogens are shed in feces by infected persons and subsequently enter the human water cycle, thus they can potentially be transmitted via water when humans come in contact with different types of water from in the urban water cycle either directly or indirectly (Figure 2). For instance, humans can be exposed to microbial pathogens in surface waters during swimming, bathing or other recreational activities. Moreover, the use of fecally polluted greywater or reclaimed water for purposes such as irrigation could potentially favor the transmission of pathogens to humans. Lastly, treatment deficiency or failure during drinking water treatment or distribution could also cause the infection of humans by excreta related pathogens present in contaminated drinking water. Thus, sanitation measures are important to safely contain and dispose human excreta (WHO, 2018), while multi barrier concepts from catchment to the consumer are required to ensure that drinking water is not fecally polluted (WHO, 2017).

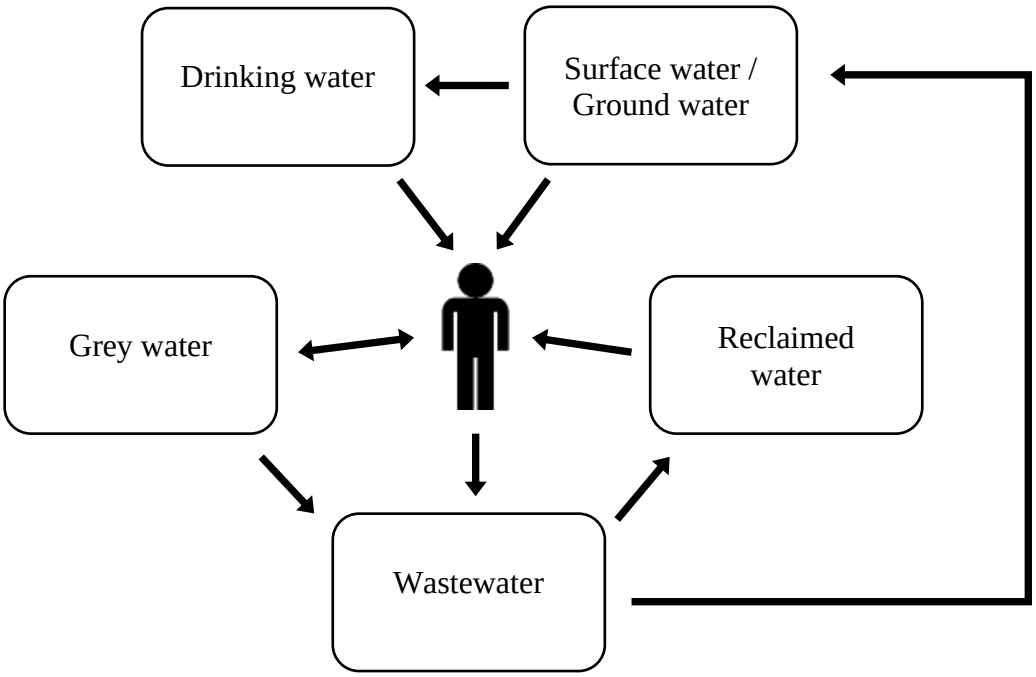

Figure 2: Simplified schematic overview of potential transmission pathways for waterborne pathogens in the urban water cycle.

1.2 Hygienic relevance of viruses in the urban water cycle

Viruses represent a huge and diverse microbial group of obligate intracellular parasites, which always require a host cell for reproduction. Potential host cells can be prokaryotic or eukaryotic cells, including bacteria, plants, animal and human cells. Viruses have a distinct host specificity, which can be broad or narrow. It is assumed that phages, which infect bacteria, have the highest abundance of all life forms on earth and contribute significantly to the carbon turnover in the oceans (Rohwer and Segall, 2015). Besides, different types of viruses can have an impact on human life either directly or indirectly. For instance, plant viruses such as tobacco mosaic viruses or pepper mild mottle viruses cause significant economic damages in agriculture around the globe.

The same accounts for animal pathogenic viruses, which can cause significant losses to farmers or – even worse – in some cases infection of humans (zoonotic viruses). Nowadays, the growing world's population and consumption of meat also increases factory farming, which enhances close contact between animals and therefore facilitates transmission of microbial pathogens between individuals. For instance, relevant pathogens in pig farming include porcine adenoviruses (PAdV), porcine circoviruses (PCV) and hepatitis E virus (HEV), which are widely detected in infected animals, feces and slurries form pig farms all over the world (Derbyshire and Brown, 1978; Vasickova et al., 2009; Yang et al., 2003; Zhou et al., 2016). PAdV mainly causes gastrointestinal and respiratory illnesses in infected pigs, whereas HEV causes asymptomatic infections in animals, but can be transmitted to humans by consumption of contaminated meat, causing severe symptoms such as liver damage. Moreover, porcine reproductive and respiratory syndrome viruses (PRRSV) are relevant in pig farming, they cause reproductive failure and respiratory illnesses, which result in high economic loss for farmers (Nathues et al., 2017). While it is known that most of these viruses can be transmitted via the fecal-oral pathway or direct contact, the role of the urban water cycle in transmission of animal pathogenic viruses is widely unknown (Fong and Lipp, 2005).

Besides animal viruses, considerable efforts are undertaken to examine and prevent transmission of human pathogenic viruses via water. The group of excreta-related pathogens includes several human enteric viruses (Table 1), which are particularly important in water hygiene because they have a low infective dose and a higher environmental persistence compared to bacteria. Enteric viruses include enteroviruses, hepatoviruses, adenoviruses, caliciviruses, astroviruses and reoviruses, their main transmission pathway is ingestion, while some viruses can also be transmitted via inhalation (adenoviruses) or direct contact

(rotaviruses). Most of the enteric viruses cause viral gastroenteritis, some can cause more severe illnesses such as hepatitis or illness of the central nervous system (e.g. poliomyelitis, meningitis). Typically, they are transmitted via the fecal-oral route, including the transmission via water directly (e.g. drinking water, bathing water) or indirectly (e.g. irrigation water). It was shown that raw vegetables can act as vector for transmission of enteric viruses when they are irrigated with contaminated water (Cheong et al., 2009). Enteric viruses reproduce in the human intestine, are shed with feces in high concentrations of up to 10^{15} viruses per gram stool by infected persons (Haramoto et al., 2018; WHO, 2018) and subsequently enter the urban water cycle.

Table 1: Characteristics of excreta-related viral pathogens (Modified after WHO, 2018).

Virus	Health significance	Transmission pathways	Important animal source	Likely importance of sanitation for control	Concentration excreted in faeces	Duration of excretion
Adenovirus	A large group of distinct viruses that cause a variety of conditions. Genotypes 40 and 41 predominantly cause gastroenteritis in children, resulting in prolonged diarrhoea (up to 10 days).	Person-to-person, through both faecal-oral and droplet transmission.	None – strict human pathogen	Low	10^{11}/g (lower with non-enteric adenovirus)	Months after symptoms resolve
Astrovirus	Common cause of diarrhoea globally, especially in young children.	Predominantly person-to-person, potentially waterborne. Outbreaks usually occur in institutional settings.	None – strict human pathogen	Low	10^{2} – 10^{15}/g	Up to two weeks after symptoms end
Enterovirus	Large number of viruses with a vast array of clinical symptoms (including poliovirus – see below).	Person-to-person and environmental exposure	None known	Uncertain	up to 10^{6}-10^{7}/g	10 days to 2 months
Hepatitis A virus	Causes acute, usually self-limiting hepatitis. Occasionally associated with death from acute liver failure.	Food and waterborne; person-to-person. Both routes can lead to outbreaks.	No (non-human primates have been infected in studies but are not part of the transmission cycle).	Medium	Prevalence in stool higher before symptoms.	Present from 14-21 days before onset to 8 days after appearance of jaundice.
Hepatitis E virus	Can cause acute hepatitis; genotype 1 associated with maternal mortality in low- and middle-income countries due to acute liver failure.	Genotypes 1 and 2 dominate in LMIC and are predominantly waterborne. Genotypes 3 and 4 dominate in Europe and are associated with consumption of contaminated pork or game meat.	Genotypes 1 and 2: no known animal transmission pathway. Genotypes 3 and 4 are zoonotic, strongly linked with pork consumption.	Medium	10^{5}/ g	1 week before symptoms up to 4 weeks following.

Table 1: Continued.

Virus	Health significance	Transmission pathways	Important animal source	Likely importance of sanitation for control	Concentration excreted in faeces	Duration of excretion
Norovirus	Leading cause of gastroenteritis outbreaks (characterized by diarrhoea, vomiting and stomach pain) in all age groups.	Predominantly person-to-person through both faecal-oral and droplet transmission; can be spread through food and water. Major cause of sporadic outbreaks in hospitals, nursing homes and other institutional settings.	None – strict human pathogen	Low	10^{11}/ g	8–60 days
Poliovirus	Acute poliomyelitis is frequently asymptomatic. A small proportion of people will develop paralysis.	Person-to-person. Some outbreaks have been associated with breakdown in sanitary infrastructure (e.g. during war)	None – strict human pathogen	Medium	—	—
Rotavirus	Major cause of acute gastroenteritis in infants globally. Common symptoms include severe watery diarrhoea, vomiting, fever and abdominal pain. Rotavirus infection is associated with severe dehydration and occasionally death.	Person-to-person.	Most rotaviruses are strict human pathogens; group C rotavirus may be associated with cattle.	Low	10^{10}–10^{12}/ g	2 days before to 10 days after symptomatic illness.
Sapovirus	Cause of acute diarrhoea and vomiting globally.	Predominantly person-to-person through both faecal-oral and droplet transmission; can be spread through food and water.	None – strict human pathogen	Low	—	—

The occurrence of enteric viruses in wastewater and surface water was investigated extensively in numerous studies before. In wastewater, enteric viruses are observed with high concentrations, typically ranging from 10^4 to 10^9 genome equivalents (gen.eq.) per liter (Farkas et al., 2018; Haramoto et al., 2018; Hata et al., 2013; Sidhu et al., 2013). Moreover, they were detected in greywater of single households, which consists of water from washing machines and bathroom greywater (O'Toole et al., 2012). Although treatment reduces the concentration in wastewater by 1-3 log units (Haramoto et al., 2018), enteric viruses are not eliminated during wastewater treatment. Thus, they are frequently detected in wastewater treatment plant effluents (Carducci et al., 2008; Cheng et al., 2012; Hata et al., 2013; Jurzik et al., 2015; Kitajima et al., 2014; Simmons and Xagoraraki, 2011) and are therefore often discharged into surface waters. Besides, the use of reclaimed water for irrigation increases the risk of crop contamination (Lopez-Galvez et al., 2016) and thus potentially facilitates transmission of viruses to humans. The detection of viruses in rivers and lakes can generally be explained not only by effluents from wastewater treatment plants, but also by combined sewer overflows, runoff from agricultural areas or wildlife (Rechenburg et al., 2006) and remobilization of pathogens from surface water sediments (Garcia-Aljaro et al., 2017; Muirhead et al., 2004). Hata et al. (2014) showed that rainfall events and subsequent combined sewer overflows contribute substantially to the viral load in river water. However, wastewater treatment plant effluents are regarded as the main source of enteric viruses in urban rivers (Prevost et al., 2015). Typically, enteric viruses can be found in surface water with maximum concentrations of 10^5 genome equivalents per liter (Cooksey et al., 2019; Elmahdy et al., 2016; Farkas et al., 2018; Hamza et al., 2009; Hata et al., 2018; Sedji et al., 2018), potentially posing a health risk for humans when surface waters are used for recreational activities. There is evidence that enteric viruses cause the major part of the environmental burden of disease caused during bathing (Timm et al., 2016), therefore this transmission route should not be neglected when assessing the role of viruses in the urban water cycle. In many cases, surface waters also serve as raw waters for drinking water production. During treatment, viruses must be either removed or inactivated to prevent a transmission to the consumer. Several studies indicated that common treatment processes including river bank filtration, slow sand filtration or flocculation are effective tools for virus removal from raw water (Bauer et al., 2011; Payment, 1991; Sprenger et al., 2014). For instance, Bauer et al. (2011) described up to 3.2 log units removal of K13 and somatic coliphages during slow sand filtration. In addition to that, ozonation and common disinfection

by UV irradiation and chlorination were also found to effectively inactivate enteric viruses (Prevost et al., 2016; Thurston-Enriquez et al., 2005). For example, disinfection with chlorine or UV radiation is suitable to inactivate viruses by several log units, however chlorine-resistant phages were found to be efficiently inactivated by 3-5 log units only when a combination of both disinfection techniques was used (Zyara et al., 2016). Depending on the source of the raw water, drinking water treatment includes a combination of several treatment steps according to the multi-barrier concept in order to ensure the production of safe drinking water, resulting in total reduction or inactivation of viruses by more than 5 log units (Albinana-Gimenez et al., 2009).

Nevertheless, several outbreaks of viral gastroenteritis were linked to fecally contaminated drinking water worldwide. The most relevant causes include a contamination of raw water in combination with treatment deficiencies (Carrique-Mas et al., 2003; Giammanco et al., 2014; Scarcella et al., 2009; Vantarakis et al., 2011), failure of drinking water treatment (Fretz et al., 2005; Kukkula et al., 1999) and contamination of drinking water reservoirs or distribution systems (Laine et al., 2010; van Alphen et al., 2014). In some cases, cross-connections between drinking water and grey water (Fernandes et al., 2006) or industrial use water (Altzibar et al., 2014) were determined as cause of an outbreak. For instance, in 2008, a massive outbreak of viral gastroenteritis with 1,699 reported cases occurred in Podgorica (Montenegro) after the drinking water was fecally contaminated during distribution (Werber et al., 2009). In this outbreak, the estimated number of affected individuals was 10,000 – 15,000, six different genotypes of human norovirus were detected. In Finland, an inappropriate cross-connection between sewage and drinking water pipelines caused a gastroenteritis outbreak with an estimated number of 8,453 cases (Laine et al., 2010). Among other microbial pathogens, noroviruses and rotaviruses were detected in water samples and feces of infected persons. It is worth to note that outbreaks of viral gastroenteritis are not only limited to the so-called developing countries and thus show that also countries with proper drinking water production and distribution require measures to strictly separate drinking water from fecally polluted waters that occur in the urban water cycle.

In order to develop concepts for the prevention of waterborne transmission of human viruses, fundamental understanding of both virus prevalence and behavior in the urban water cycle is required. Because handling and quantification of human enteric viruses have several drawbacks including the need for special laboratory equipment and trained personal, many studies focused on the use of surrogate organisms in order to study the behavior of enteric

viruses in the aquatic environment. Surrogates include bacteriophages or animal pathogenic viruses that are structurally similar to their corresponding human viruses (e.g. murine noroviruses). In 2014, German Environment Agency recommended the quantification of coliphages in raw water to characterize the microbial pollution in raw water for drinking water production (Umweltbundesamt, 2014). One major advantage is that bacteriophages are comparatively easy to handle, can be propagated and quantified using their respective host bacterium and do not require eukaryotic cell lines. The most commonly used group of bacteriophages are coliphages, which infect *E. coli* and related species of the genera *Klebsiella* and *Shigella* (Jofre et al., 2016). There is a variety of coliphages with different modes of infection. Typically, two defined subgroups, namely somatic and F-specific coliphages, are defined and can easily be detected with plaque assays using bacterial host strains. The use of certain strains however implies that the coliphages are capable of infecting the specific strain, which selects only a fraction of phages for enumeration. Therefore, results can differ depending on the host strain which is used for quantification. The application of surrogate viruses is controversially discussed in literature since many years, as reviewed by Jofre et al. (2016). In water, coliphages are often used as surrogates for the presence of enteric viruses (Haramoto et al., 2005; Jiang et al., 2001; Vergara et al., 2015), for virus removal and the evaluation of inactivation processes during water treatment (Bauer et al., 2011; McMinn et al., 2017; Meschke and Sobsey, 2003; Szewzyk et al., 2006) or for microbial source tracking (Lee et al., 2011; Plummer and Long, 2007). Moreover, coliphages in water are often discussed as indicator organisms for a fecal pollution (McMinn et al., 2017; Muniesa et al., 2018). Typically, indicators should not reproduce outside the animal host, correlate with human pathogens of fecal origin and display a similar survival profile compared to the respective pathogens (Field and Samadpour, 2007). Although it is undisputed that the environmental behavior and persistence of enteric viruses might be better displayed by bacteriophages than by indicator bacteria, variations in virus size, genome type and structure of the viral capsid imply that there might also be differences concerning stability and persistence in the environment. Consequently, many studies showed that the presence of coliphages does not necessarily correlate with that of relevant human pathogenic viruses (Hot et al., 2003; Jiang and Chu, 2004; Jiang et al., 2007; Jurzik et al., 2010; Westrell et al., 2006). Moreover, some authors have argued that coliphages might propagate under certain conditions in the aquatic environment. For instance, Borrego et al. (1990) found that coliphage propagation in seawater is possible at 36 °C, however no propagation was observed

at 18 °C. In another study, the authors proposed that coliphage multiplication in estuarine waters might occur at lower temperatures in presence of bacterial host cells (Vaughn and Metcalf, 1975). Seeley and Primrose (1980) isolated different types of coliphages from river water and concluded that common phage isolation with incubation temperatures of 36 °C excludes the detection of low temperature phages, which in turn might propagate in natural waters. In contrast to that, other more recent studies reported that a propagation of coliphages in aquatic environments is unlikely or negligible (Jofre et al., 2016; Muniesa et al., 2003; Woody and Cliver, 1995). It is unclear, whether coliphages are appropriate models to study the interaction of enteric viruses with aquatic biofilms in the urban water cycle.

1.3 Aquatic biofilms as reservoirs for viruses

Aquatic biofilms are ubiquitous microbial aggregates, embedded in a matrix of self-produced extracellular polymeric substances (EPS), forming at surfaces that are in contact with non-sterile water (Hall-Stoodley et al., 2004). They can be found in all natural and technical aquatic systems, representing complex and diverse ecosystems which are important microbial habitats in the urban water cycle. Biofilm processes are widely applied for the purification in wastewater treatment using biofiltration or biofilm reactors (Kim et al., 2009; Smith et al., 2015; Zhang et al., 2016). Moreover, biofilms play an important role in processes such as slow sand filtration (Pfannes et al., 2015) or activated carbon filtration (Velten et al., 2011; Zhang et al., 2011) during drinking water production. Aquatic biofilms also form on stones or sediments in surface waters, in water pipes or on plumbing materials of a drinking water distribution system (Camper et al., 1998; Chen et al., 2004; LeChevallier et al., 1987), diverse in shape and composition. For instance, Moritz et al. (2010) characterized growth of drinking water biofilms on different materials and found total cell counts of 10^4 to 10^7 cell/cm^2, within 14 d of growth, depending on the type of surface material. In river biofilms, total cell counts of up to 4.9 x 10^{12} cells/100 g (wet weight) were observed (Balzer et al., 2010), showing that aquatic biofilms in natural systems are densely polulated microbial habitats. Biofilms can harbor fecal bacteria (Balzer et al., 2010), act as reservoir for microbial pathogens (Abraham, 2011), protect microorganisms from disinfection and thus play an important role in water hygiene, as reviewed by Wingender and Flemming (2011) and Nocker et al. (2014).

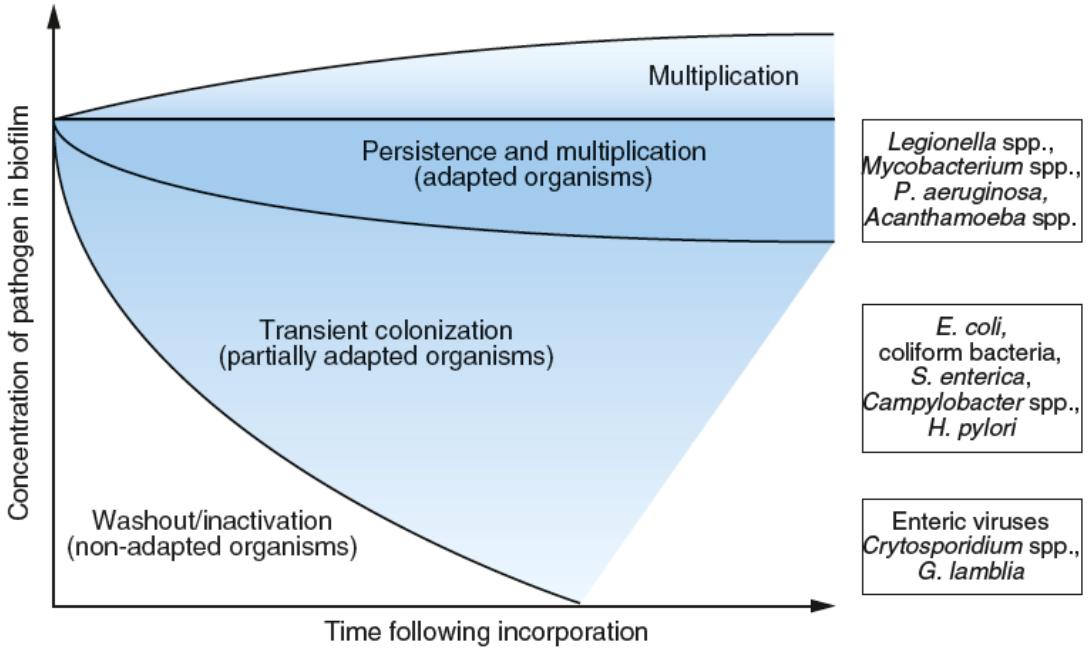

Figure 3: Potential fate of microbial pathogens in biofilms. (Wingender, 2011)

The fate of microbial pathogens in aquatic biofilm depends on their individual potential to multiply or persist within the biofilm (Figure 3). While growth of some hygienically relevant bacteria (e.g. *Pseudomonas aeruginosa*, *Legionella* spp.) in biofilm environments is known (Declerck et al., 2009; Kuiper et al., 2004; Wingender, 2011), other pathogens including human enteric viruses cannot multiply outside their human host and thus can only enter the biofilms from the bulk water. Thus, the fate of enteric viruses depends on the initial concentration in the biofilm, the detachment rate (washout) and processes of inactivation.

Although most viruses can't multiply in biofilms, the interaction between viruses and aquatic biofilms is very complex and includes a variety of processes. Viruses can generally adsorb to different biotic and abiotic surfaces such as clays, sediments or particulate matter (Gerba, 1984), potentially also including biotic and abiotic surfaces found in aquatic biofilms. The EPS mainly consist of polysaccharides, proteins, nucleic acids and lipids (Figure 4), which generate a variety of attractive and repulsive forces, including electrostatic interactions, van der Waals interactions or hydrogen bonding (Flemming and Wingender, 2010). Thus, the complex matrix also harbors sorption sites which are potentially available for the retention of nanoparticles (Flemming, 1995), which implies that also biological particles such as viruses could be adsorbed. It was found that nanoparticles initially adsorb to biofilm components through combinations of electrostatic, steric and hydrophobic interactions, before they migrate deeper into the EPS matrix by diffusion (Ikuma et al., 2015). Lacroix-Gueu et al.

(2005) studied the transport of C2 bacteriophages into mucoid biofilms of *Stenotrophonas maltophilia* and demonstrated that the phages were able to penetrate into the EPS matrix of the biofilm.

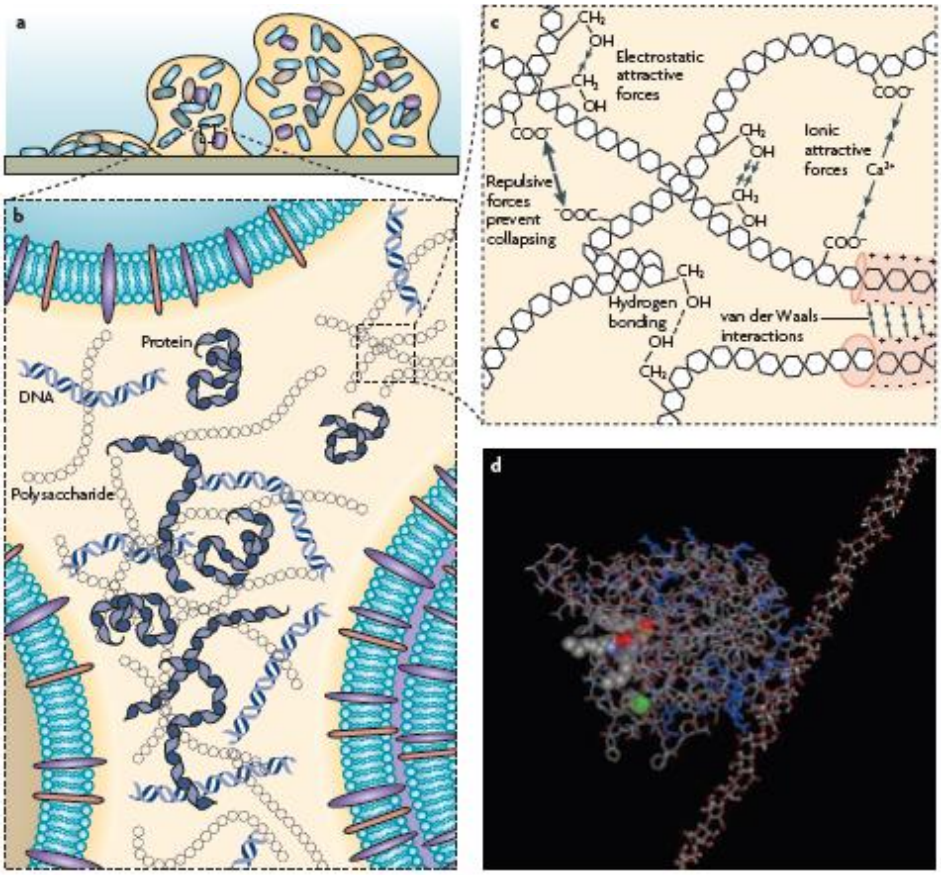

*Figure 4: Schematic structure of an aquatic biofilm in different dimensions (**a-c**) and molecular modelled structure of the interaction between alginate and lipase in a biofilm by P. aeruginosa (**d**). (Flemming and Wingender, 2010)*

The mechanistic aspects of interaction between aquatic biofilms and viruses still are not fully understood, but there several studies which investigated the influence of numerous physicochemical parameters on the adsorption of viruses to biotic and abiotic surfaces. Although most studies described processes of virus adsorption during slow sand filtration, groundwater recharge or similar water treatment applications, the basic physicochemical processes of virus adsorption might also be valid for aquatic biofilms at least with restriction, even though biofilms obviously pose a different type of matrix compared to sand or soil. Adsorption was shown to be influenced by a variety of factors including characteristics of the adsorbate, the adsorbent and several environmental parameters. Studies elucidated the effects of pH (Dowd et al., 1998; LaBelle and Gerba, 1979), salinity or ionic strength (Cao et al., 2010; LaBelle and Gerba, 1979; Lance and Gerba, 1984a), adsorbent material (Cao et al.,

2010; Wang et al., 1981), -porosity (Chrysikopoulos and Aravantinou, 2014; Wang et al., 1981), and virus type (Dowd et al., 1998; LaBelle and Gerba, 1979; Wang et al., 1981) on virus adsorption. Moreover, temperature (Chrysikopoulos and Aravantinou, 2014), hydraulic conditions (Chrysikopoulos and Aravantinou, 2014; Lance and Gerba, 1984b; Van Cuyk and Siegrist, 2007; Vaughn et al., 1981), oxygen content (Frohnert et al., 2014), presence of clays (Chrysikopoulos and Aravantinou, 2014) or organic substances (Aronino et al., 2009; Cao et al., 2010; Lance and Gerba, 1984a; Van Cuyk and Siegrist, 2007) in water were found to influence adsorption of viruses. Dowd et al. (1998) reported that the type of virus has an impact on the adsorption, because both size and isoelectric point (IEP) influenced virus removal in soil columns. The presence of organics in soil and water revealed different conclusions in the literature. While Cao et al. (2010) and Aronino et al. (2009) stated that the removal of viruses from water during sand filtration is low for water containing a high organic load, others found the opposite effect (Lance and Gerba, 1984a; Van Cuyk and Siegrist, 2007). Besides adsorption, also desorption has been demonstrated in the literature. For instance, Lance et al. (1976) and Quanrud et al. (2003) have shown that adsorbed viruses can be remobilized during periods of rainfall because the ionic strength of rainwater is low compared to treated wastewater effluent.

Although most of the studies mentioned above regarded virus adsorption to be a process between virus particles and abiotic surfaces, it can be assumed that also biofilms contributed to the retention of viruses in many cases, although it was not assessed. In column experiments, Heistad et al. (2009) applied septic tank effluent spiked with phage PRD-1 to filters packed with porous filter material (Filtralite®). It turned out that the removal of phages was enhanced after higher operation time. The authors proposed that biofilm formation increased surface area and thus provided adsorption sites within the filter. Similarly, Van Cuyk and Siegrist (2007) performed adsorption experiments with MS2 and PRD-1 phages in small sand columns supplied with simulated groundwater or septic tank effluent. The authors demonstrated that the retention of viruses increased with increasing time of operation, suggesting that biofilms in the sand column enhanced adsorption (Van Cuyk and Siegrist, 2007). Interestingly, there is also literature showing that virus retention during soil passage can be low if adsorption sites are depleted after long operation time of soil filters (Schaub and Sorber, 1977), which could mean that also the adsorption capacity of aquatic biofilms is limited.

The adsorption of viruses to aquatic biofilms can affect the persistence of viruses in the aquatic environment. Several studies demonstrated that viruses which adsorbed to surfaces are protected from inactivation (Chung and Sobsey, 1993; Hurst et al., 1980; Sakoda et al., 1997; Smith et al., 1978). Hejkal et al. (1981) reported that particle-associated viruses are protected against chlorination. Once entrapped in the biofilm matrix, viruses might also benefit from protection towards environmental stressors such as desiccation or antimicrobial agents (Lacroix-Gueu et al., 2005).

Not only physicochemical processes, but also biological interactions might influence the fate of viruses in biofilms. Aquatic biofilms represent a complex microbial habitat with a high density of various organisms. Potentially, viruses could not only interact with the EPS, but also with bacterial cells or other organisms. For instance, free-living amoebae, which are frequently found in aquatic systems (Hsueh and Gibson, 2015; Loret and Greub, 2010; Scheid and Schwarzenberger, 2012), could influence the retention and protection of viruses in biofilms. Amoebae graze on biofilms, where they could potentially internalize viruses, subsequently providing protection against environmental stressors. Authors described the internalization of viruses such as coxsackie B viruses or adenoviruses by amoebae (Hsueh and Gibson, 2015; Mattana et al., 2006; Scheid and Schwarzenberger, 2012) and the presence of viruses or viral DNA in naturally occurring amoebae (Lorenzo-Morales et al., 2007; Staggemeier et al., 2016). To date, there are no studies which investigated the role of amoebae for the persistence of viruses in aquatic biofilms.

Biological interactions might not only affect the persistence of viruses in biofilms, but some viruses can also have an influence on the biofilm itself. For instance, there is evidence that bacteriophages can enhance environmental fitness and tolerance to stressors of biofilm bacteria because they can promote horizontal gene transfer between their hosts organisms in presence of a selective pressure (Jin et al., 2012). Thoulouze and Alcover (2011) proposed that T-cell leukemia virus type 1 (HTLV-1) induces the formation of an extracellular carbohydrate-rich matrix by infected cells in order to form “viral biofilms”, which in turn could increase the probability of infecting neighboring cells. On the other hand, phages can induce lysis of their respective host bacteria or destabilize the surrounding EPS by phage-encoded depolymerase enzymes, which can disrupt the biofilm structure (Sutherland et al., 2004). In case of enteric viruses however, the respective host cells are not present in the aquatic environment, thus it is unlikely that enteric viruses would affect the structure of biofilms.

In conclusion, biofilms represent a complex and permanently altering environment, consequently there is a variety of physicochemical and biological factors that might influence the fate of viruses.

1.3.1 Approaches to study viruses in aquatic biofilms

The interaction between viruses and aquatic biofilms in the urban water cycle complex and depends on a variety of factors, as described above. Presumably, it is not possible to generalize the behavior of viruses in different natural and technical systems. In several studies, researchers have investigated the fate of viruses in different aquatic biofilms, typically using two different approaches:

In field studies, samples are taken from technical or natural systems and subsequently analyzed for the occurrence of enteric viruses or respective surrogates in water and biofilms. In many cases, aquatic biofilms are removed from surfaces by mechanical means (e.g. scraping, ultrasonication) and further analyzed in the laboratory. The occurrence of selected target viruses can either be determined qualitatively or quantitatively in order to determine the distribution between biofilm and bulk water. On the one hand, this approach represents a realistic scenario without artificially grown biofilms. On the other hand, field studies can only display a snapshot of the system at the sampling event, which is likely to be influenced by a variety of environmental parameters that cannot be controlled as in a laboratory-scale system. For instance, it is possible that the concentration of target viruses in water varies significantly over time, which could create wrong conclusions about the distribution between water and biofilm. Although this approach can help to monitor the pathogens in a real-world system, it is not the most common tool to investigate the interaction between viruses and aquatic biofilms.

In model studies, experiments are carried out under distinct conditions in the laboratory that can help to understand the basic mechanisms of virus/biofilm interactions. Model studies can be performed using selected enteric viruses, surrogates, or both. In contrast to field studies, physicochemical or biological parameters can be controlled to test certain hypotheses. However, model studies require the transfer of the results to practical situations, but laboratory-scale experiments are not always realistic to simulate a real-world system.

Regardless of the approach used, adequate purification or concentration methods are required in order to quantify viruses in biofilms. Initially, viruses that are bound to the complex matrix

of aquatic biofilms and must be separated from the matrix prior to analysis, without impairing the viral infectivity. Most methods rely on both physical and chemical separation, including stirring, vortexing (Helmi et al., 2008; Skraber et al., 2009a), ultrasonication (Helmi et al., 2010; Nazir et al., 2011) or a combination of several techniques (Botzenhart and Hock, 2002; Quignon et al., 1997b; Wellings et al., 1975). On the contrary, Storey and Ashbolt (2002) demonstrated that stomaching inactivates 32 % of B40-8 phages and 20 % of MS2 phages within 2 minutes, and thus is not appropriate to remove biofilms from stainless steel and PVC coupons. For chemical separation, different elution buffers are used to disrupt electrostatic interactions (e.g. by alkaline pH) between viral particles and biofilms. These buffers often contain organic substances such as glycine, beef-extract or skim milk (Botzenhart and Hock, 2002; Helmi et al., 2008; Nazir et al., 2011; Skraber et al., 2009a), which compete with binding sites for virus adsorption (Gerba, 1984). Similar elution buffers were also successfully used for elution of viruses from other complex sample matrices such as mud, soil and treated biowaste (Deboosere et al., 2012; Guzmán et al., 2007).

Besides efficient virus elution, it is also important to purify viruses from the eluate to prevent inhibition of downstream detection methods. Therefore, most authors applied additional concentration/purification steps after initial virus elution. Methods include ultracentrifugation, ultrafiltration or polyethylene glycol (PEG) precipitation. The fact that biofilms are diverse in structure and composition implies that the efficiency of virus elution depends on the individual sample matrix. Presumably, there is no perfect purification method for all sample types.

Typical detection methods include molecular biology methods (e.g. quantitative real-time PCR, Next-Generation Sequencing), culture-based methods (e.g. cell culture), or combinations of both (e.g. integrated cell culture-PCR). The most common method is the quantitative real-time PCR, which has the major drawback that it does not distinguish between infectious and non-infectious viruses and therefore includes false-positive detection of free nucleic acids or DNA/RNA from damaged viruses. Recently, a modified qPCR method (capsid-integrity qPCR) was described, which can reduce false-positive qPCR results for some applications (Leifels et al., 2015; Prevost et al., 2016). This method is based on the use of ethidium monoazide (EMA) or propidium monoazide (PMA), which can only enter damaged viral capsids, while intact capsids are impermeable. EMA and PMA subsequently intercalate into nucleic acids and hinder PCR amplification and thus detection by qPCR. Unfortunately, the method does not exclude false-positive results from non-infectious viruses

with a damaged genome but intact capsid (Leifels et al., 2015). To date, culture-based methods remain the gold standard for quantification of infectious enteric viruses. Cultivation methods for enteric viruses rely on infection of distinct eukaryotic cell lines by the specific type of virus and the subsequent observation of morphological changes or apoptosis of the cells (cytopathic effects). However, cell culture is relatively expensive, time consuming and not applicable for all viruses (Hamza et al., 2011b). Particularly, the lack of a suitable cell line for detection of human noroviruses made it impossible to quantify one of the most relevant enteric viruses in the past. For this reason, murine noroviruses were widely used instead of human noroviruses to study norovirus behavior, as reviewed by Wobus et al. (2006). Recently, Ettayebi et al. (2016) reported a potential candidate cell line for the detection human pathogenic norovirus strains.

Besides the commonly applied methods mentioned above, some studies used assays based on fluorescence microscopy (Noble and A., 1998) or flow cytometry (Brussaard et al., 2010; Chen et al., 2001; Deng et al., 2012; Lambeth et al., 2005; Weaver and Kadan, 2000) for virus detection. Also confocal laser scanning microscopy was discussed as a powerful tool for virus detection in complex samples as reviewed by Weinbauer et al. (2009), however unspecific staining of nucleic acids can cause false positive results and therefore these methods should be used with care. Advantages and drawbacks of recent detection methods for enteric viruses in environmental water samples were recently reviewed elsewhere (Haramoto et al., 2018).

1.3.2 Occurrence of viruses in surface water biofilms

Surface waters such as rivers and lakes are complex aquatic environments that can differ tremendously in respect of size, structure, intended use, anthropogenic influence and thus viral load. As described before (1.1), many surface waters worldwide receive either treated or untreated wastewater, are polluted by combined sewer overflows, surface runoff from agricultural areas or wild animal excretions. Because surface waters are often occupied for recreational purposes such as bathing and swimming, or used as raw waters for drinking water production, the hygienic quality of is often monitored by quantification of bacterial and viral indicators and – in some cases – the detection of viral pathogens. However, directives and guidelines mostly focus on the presence of microorganism in water, whereas biofilms are not considered, although they might represent an important reservoir for fecal indicators and different pathogens. Surface water biofilms, either on stones (epilithic biofilms) or sediment particles in the riverbed could take up viruses from the water phase and release them again

after a certain residence time. The role of marine and estuarine sediments for the retention of enteric viruses was discussed in several studies, as recently reviewed by Hassard et al. (2016). On the contrary, only few studies have addressed the occurrence of viruses in fresh water biofilms until now. Investigating the fate of coliphages, Skraber et al. (2009b) found that fresh water sediments contained phages even though the corresponding water sample was tested negative, moreover the authors demonstrated that the retention depended on the type of virus. In another study, enteroviruses were found in river sediment and the overlying water, whereby enteroviruses accumulated in sediments by a factor of four (Ali et al., 2004). In a more recent study, Elmahdy et al. (2016) showed that group A rotavirus and human adenoviruses can be detected in surface water sediments, interestingly the concentration of human adenoviruses was two or three orders of magnitude higher than in the bulk water. Garcia-Aljaro et al. (2017) also found that river water sediments harbor different microorganisms including fecal indicators and pathogens such as enteroviruses. Moreover, they showed that the microbial load of sediments increases after rainfall events and subsequent fecal pollution of the water body. The dependency of virus concentrations in the riverbed from concentrations in water was also proposed by Hirotani et al. (2013) for some groups of coliphages. It was found that fluctuations of the phage concentrations in river biofilms correlated with fluctuations in the bulk water, although that was not the case for all phages tested (Hirotani et al., 2013). Besides, the different occurrence of viruses in surface water biofilms and sediments could also be attributed to differences in persistence within the biofilm or sediment (Elmahdy et al., 2018). In artificial microcosms, Elmahdy et al. (2018) described that a significant transfer of human adenoviruses (HAdV) to sediments within 24 h was observed in all microcosms tested, while a similar pattern was not always found in case of murine noroviruses (MNV). The authors suggested that factors such as virus type, virus shape, surface charge or hydrophobic interactions might cause differences in deposition rates between different types of viruses.

The fact that viruses can be trapped on surfaces within rivers or lakes arises the question, whether biofilms and sediments should be included when evaluating the microbiological quality of surface waters. However, since the mechanisms and kinetics of virus adsorption and desorption depend on various factors (as mentioned above), it is difficult to include the potential influence of surface water biofilms and sediments in quantitative microbial risk assessment. To date, it is not clear, whether aquatic biofilms generally contribute significantly to the risk for waterborne transmission of viruses in the urban water cycle. There is a need for

quantitative data about virus prevalence in aquatic biofilms of natural systems to better understand the fate of viral pathogens in the urban water cycle.

1.3.3 Occurrence of viruses in drinking water biofilms

It is known that biofilms are formed on surfaces of piping materials, fittings and sealings in drinking water distribution systems and that they can be a reservoir for a variety of hygienically relevant microorganisms, as reviewed by Wingender (2011). In contrast to surface water, drinking water must not contain human pathogenic viruses in concentrations that would pose a risk to human health to prevent a waterborne transmission to the consumer. Consequently, enteric viruses are not expected to occur in drinking water biofilms, unless a fecal contamination had occurred. Besides, small leakages in a distribution network could potentially cause a contamination of drinking water with polluted water without inducing an outbreak, which could lead to an accumulation of pathogens in biofilms. Under normal operation, the water pressure prevents the intrusion of contaminated water into the distribution system, but it was shown that this is might not be sufficient in case of short negative pressure events (Teunis et al., 2010).

Several viral outbreaks in the past were linked to the consumption of fecally contaminated drinking water (Hewitt et al., 2007; Kukkula et al., 1999; Scarcella et al., 2009; Werber et al., 2009), with most outbreaks being caused by cross-connections between drinking water and wastewater. However, the role of drinking water biofilms was not investigated during these outbreaks. In a case study, Miettinen et al. (2012) investigated a waterborne outbreak of gastroenteritis caused by a fecal contamination of the distribution network in Nokia (Finland). Due to an undesired connection between a wastewater line and a drinking water line in a wastewater treatment plant, wastewater entered the distribution system, consequently 8,453 cases of gastroenteritis were reported during the outbreak. Although the cause was found and rectified shortly after first cases were reported, human adenoviruses were detected in drinking water for a period of more than two months, whereas bacterial indicators were not found. Finally, the continuous recontamination of the bulk water was linked to biofilms that trapped viruses in the distribution network (Miettinen et al., 2012).

A few laboratory studies assessed the interaction of viruses with artificially grown drinking water biofilms on different materials. Storey and Ashbolt conducted several studies showing that bacteriophages (B40-8, MS2, φX174) are incorporated into drinking water biofilms that

were grown for 72 h or 3 months on stainless steel or uPVC (Storey and Ashbolt, 2001; Storey and Ashbolt, 2003a; Storey and Ashbolt, 2003b). In 3-month-old biofilms, coliphage φX174 was still detected via plaque assay after a period of 20 d, whereas B40-8 and MS2 retained infectivity for the entire period of sampling (30 d) (Storey and Ashbolt, 2003a; Storey and Ashbolt, 2003b). Lehtola et al. (2007) showed that canine calicivirus concentrations in drinking water biofilms grown on PVC decreased by 70 % within three weeks after incorporation, using quantitative real-time PCR. Besides caliciviruses, also poliovirus type 1 can persist in drinking water biofilms on PVC for at least 24 h (Quignon et al., 1997a; Quignon et al., 1997b). Interestingly, Pelleieux et al. (2012) found that the formation of biofilms can increase the adsorption of Qβ phages to HDPE pipes, whereas the affinity of MS2 and GA to the biofilms on HDPE was lower compared to clean HDPE. Apparently, retention differed significantly between different types of viruses. In another study, bacteriophages (MS2, φX174) and poliovirus type 1 were incorporated into 7-month-old drinking water biofilms grown on polycarbonate (Helmi et al., 2008). Infectious viruses were detected in biofilms for maximally 6 d after incorporation, while poliovirus RNA was detected throughout the whole period of sampling (34 d), indicating inactivation of polioviruses over time. Obviously, most of the studies mentioned above performed laboratory experiments with surrogate viruses in order to test a potential accumulation of viruses in drinking water biofilms. It remains unclear, whether bacteriophages are appropriate models to study the interaction between enteric viruses and drinking water biofilms.

1.3.4 Occurrence of viruses in wastewater biofilms

Municipal wastewater contains a variety of different chemical and microbial pollutants, including human pathogenic viruses, that are transported to wastewater treatment plants (Farkas et al., 2018; Haramoto et al., 2018; Hata et al., 2013; Sidhu et al., 2013). The interaction between viruses and wastewater biofilms can either be beneficial (e.g. when viruses are removed from wastewater by biofilm processes) or undesired (e.g. when virus inactivation is inhibited due to protection within the biofilm). Only a few studies have demonstrated that enteric viruses can attach to wastewater biofilms (Helmi et al., 2008; Skraber et al., 2007; Skraber et al., 2009a), from where they can be released to the bulk water after a certain time. Skraber et al. (2009a) found that enteroviruses, noroviruses and F-specific coliphages were present in natural wastewater biofilms, although they were not always detected in the corresponding water sample, which implies the retention in the biofilm.

Additionally, the authors performed persistence experiments and showed that the decrease in virus concentration is faster in bulk wastewater than in wastewater biofilms. Consequently, the increased persistence in biofilms might affect the prevalence of viruses in wastewater and could lead to dispersal of viruses in non-epidemic periods (Skraber et al., 2009a). This could be especially relevant for viruses such as noroviruses, which are known to show seasonality, as reviewed by Ahmed et al. (2013).

Although the hygienic relevance of wastewater biofilms in transmission of enteric viruses to humans is presumably low compared to surface or drinking water biofilms, data in the literature indicate that they have the tendency to incorporate a variety of viruses.

2. Aims of this study

Aquatic biofilms are ubiquitous in natural and technical systems of the urban water cycle, their role as reservoirs of pathogenic bacteria is well investigated, whereas enteric viruses are widely neglected in biofilm research. Viral pathogens might become incorporated into aquatic biofilms, where they are protected against environmental stressors and might be released again into the water after certain residence time. In the literature, bacteriophages were often used as surrogate organisms to study the interaction of viruses with biofilms, however this might not always be a realistic model for the behavior of human enteric viruses. Moreover, there are only limited data about the extent of virus accumulation and the physicochemical and biological parameters which influence the interaction. The main goal of this work was to show that different types of biofilms from both natural and technical systems in the urban water cycle can serve as reservoir for relevant infectious viruses in the aquatic environment, which included the following aspects:

- Comparison and validation of different methods to elute and quantify viruses from surface water biofilms and sediments,
- Assessment of the distribution of enteric viruses, somatic coliphages and fecal bacteria and their dependency on enhanced rainfall events in water, biofilms and sediments of an urban river in Germany,
- Investigation of the occurrence of somatic coliphages and relevant animal pathogenic viruses in water and biofilms of a drinking water systems for animals from piglet breeding farms in Germany,
- Determination of the distribution of enteric viruses and coliphages between drinking water and artificially grown drinking water biofilms in a laboratory-scale model system under different hydraulic and physicochemical conditions,
- Analysis of biological interactions between a) monospecies biofilms of *E. coli* and somatic coliphages under simulated environmental conditions and b) free-living amoebae and enteric viruses in the aquatic environment.

3. Materials

3.1 Chemicals

Table 2: List of chemicals used in this study.

Chemical	Manufacturer
4′,6-diamidino-2-phenylindole	Sigma-Aldrich
Acetic acid	Fisher Scientific
Agarose for analytical nucleic acid electrophoresis	Merck
Ammonium iron(II) sulfate hexahydrate	Carl Roth
Antibiotic Antimycotic Solution (100×)	Sigma-Aldrich
AF2 Mountant solution	Citifluor
Bacto Agar	Becton Dickinson
Bacto Peptone	Becton Dickinson
Bovine serum albumin	Sigma-Aldrich
Calcium carbonate (anhydrous)	Carl Roth
Calcium chloride dihydrate	Carl Roth
Crystal violet	Merck
Dulbecco's Modified Eagle's medium	Sigma-Aldrich
Dulbecco's phosphate-buffered saline	Sigma-Aldrich
Ethanol absolute	Fisher Scientific
Fetal bovine serum	Sigma-Aldrich
Glucose	ITW Reagents
Glycerol	Merck
Glycin	Carl Roth
Hydrochloric acid (37%)	Bernd Kraft
Immersion liquid Type N	Leica
LB medium (Lennox)	Carl Roth
Magnesium chloride hexahydrate	Carl Roth
Magnesium sulfate heptahydrate	Fluka
Meat extract	Merck
Methanesulfonic acid	Merck

Table 2: Continued.

Chemical	**Manufacturer**
Polyethylene glycol 6000	EMD Millipore
Potassium phosphate, monobasic	Carl Roth
Proteose Peptone	Becton Dickinson
R2A Agar	Becton Dickinson
Rotiphorese®10x TBE buffer	Carl Roth
Skim milk powder	Fluka
Sodium carbonate	Carl Roth
Sodium chloride	Bernd Kraft
Sodium hydroxide	Bernd Kraft
Sodium phosphate dibasic dihydrate	Sigma-Aldrich
SYBR® Safe DNA Gel stain	Invitrogen
Tri-Sodium citrate dihydrate	Carl Roth
Triton X-100	AppliChem
Yeast extract	Merck

3.3 Instruments

Table 3: List of instruments used in this study.

Instrument	Type	Manufacturer
Autoclave	Typ 400E	HP Medizintechnik
Autoclave	Hiclave HV-25l	HMC Europe
Balance	ED4202S-CW	Sartorius Weighing Technology
Biological safety cabinet	Nu-437-400E	NuAire, Inc.
Biological safety cabinet	1.8 Safe 2010	Holten LaminAir
Centrifuge	5415D	Eppendorf
Centrifuge	5430R	Eppendorf
Centrifuge	Pico 21	Thermo Fisher
Centrifuge	Fresco 21	Thermo Fisher
Centrifuge	5810R	Eppendorf
Centrifuge	5804R	Eppendorf
Centrifuge	Multifuge 3 S-R	Heraeus
Digital thermometer	Ama-digit ad 30th	Amarell
Epifluorescence microscope	AxioScope.A1	Carl Zeiss MicroImaging
Fine balance	BP210S	Sartorius
Fluorometer	Qubit® 3.0	Thermo Fisher Scientific
Freezer -20 °C	GNP 3056	Liebherr
Freezer -20 °C	GS 801	Liebherr
Freezer -20 °C	UG 1211	Liebherr
Freezer -70 °C	Herafreeze HFU 586 Basic	Thermo Scientific
Fridge	Labex-432	Kirsch
Fridge	KTR 15421	Bosch
Gel documentation system	Universal Hood 2	Bio-Rad Laboratories
Gel electrophoresis unit	Typ HE33	Amersham
Heating cabinet	100-800	Memmert
Hybridization oven	HBSNSR220	Thermo Scientific
Icemachine	RF0266A	Manitowoc
Incubator (36 °C, CO_2)	CB ATP.line	Binder

Table 3: Continued.

Instrument	Type	Manufacturer
Incubator (25 °C)	ICP 500	Memmert
Incubator (36 °C)	Heratherm IMH 100	Thermo Fisher
Ionchromatography system	Dionex Aquion	Thermo Scientific
Light microscope	11020518102	Leica Microsystems
Light microscope	CKX41	Olympus
Magnetic stirrer	RH basic 2	IKA
Magnetic stirrer	RET basic	IKA
Magnetic stirrer	RO 10	IKA
Microtiterplate reader	Infinite M200 PRO	Tecan Group
Microwave oven	MM6459	Micromaxx
PCR Cycler	5345	Eppendorf
PCR Cycler	5332	Eppendorf
PCR working station	AC632LFUVC-220	AirCleanSystems
Peristaltic pump	ISM1077 & ISM185A	Ismatec
pH meter	FiveEasy F20	Metler Toledo
Photometer	Chematest 20	SWAN Analytical
Photometer	Cell density meter model 40	Fisher Scientific
Power supply for Gel electrophoresis unit	Power Pack P25	Biometra
qPCR	CFX96 Touch™	Bio-Rad Laboratories
qPCR	Rotor-Gene 6000	Corbett Research
QuantiTray Sealer	Model 2x	IDEXX Laboratories
Shaking water bath	1092	GFL
Thermomixer	5382	Eppendorf
Ultrasonic bath	RK 103H	Bandelin
Vortex	Model G-560E	Scientific Industries
Water purification system	Milli-Q, Q-POD	Merck Millipore
Waterbath	1013	GFL

3.4 Software

Table 4: List of software used in this study.

Software	Version	Manufacturer
CFX Manager™	3.1	Bio-Rad
Chromeleon	7.2 SR5	Thermo Scientific
Endnote	X7	Thomson Reuters
i-control	1.8 SP1	Tecan
Office	2010, 2016	Microsoft
OriginPro	2016G	OriginLab Corporation
Quantity-One	4.6.3	Bio-Rad
Rotor-Gene 6000 Series	1.7	Corbett Research

3.5 Commercially available kits

Table 5: List of the commercially available kits used in this study.

Kit	Manufacturer
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
QIAamp DNA Blood Mini Kit	Qiagen
QuantiTect Probe Kit	Qiagen
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific

3.6 Microorganisms and cell lines

Table 6: List of bacteria, viruses, amoebae and eukaryotic cell lines used in this study.

Organism / Cell line	Specification	Origin
Human adenovirus	HAdV-5	Prof. Dr. Überla, Ruhr-University Bochum
Murine norovirus	MNV-1	Prof. Dr. Überla, Ruhr-University Bochum
Coliphage φX174	DSM 4497	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
Escherichia coli	DSM 13127	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
Acanthamoeba castellanii	ATCC® 30234™	ATCC American Type Culture Collection
African rhesus monkey kidney (MA-104) cells	-	Prof. Dr. Überla, Ruhr-University Bochum
Human lung carcinoma (A549) cells	-	Prof. Dr. Überla, Ruhr-University Bochum
Murine macrophage (RAW 264.7) cells	-	Prof. Dr. Überla, Ruhr-University Bochum

3.7 Culture media

Lysogeny broth (LB), (Roth)

Composition: tryptone: 10.0 g, yeast extract: 5.0 g, NaCl: 5.0 g, pH: 7.0 ± 0.2. To prepare the medium, 20 g of commercially available medium were dissolved in 1 l of deionized water and autoclaved for 15 min at 121 °C. The medium was stored at room temperature in the dark for a maximum period of 6 months.

R2A, (Becton Dickinson)

Composition: 0.5 g, dextrose: 0.5 g, soluble starch: 0.5 g, sodium pyruvate: 0.3 g, dipotassium phosphate: 0.3 g, magnesium sulfate: 0.05 g, agar: 15.0 g, pH: 7.2 ± 0.2. To prepare R2A agar plates, 18.2 g of commercially available medium were suspended in 1 l of deionized water, mixed and autoclaved for 15 min at 121 °C. Agar was cooled in a water bath to 60 °C and poured into Petri dishes with 20 ml per plate. Agar plates were stored at room temperature in the dark for a maximum period of 6 months.

Modified Scholtens' Broth (MSB)

Composition: peptone: 10 g, yeast extract: 3 g, meat extract: 12 g, NaCl: 3 g, Na_2CO_3 solution (150 g/l): 5 ml, $MgCl_2$ solution (100 g $MgCl_2$ x 6 H_2O in 50 ml water): 0.3 ml. After addition of 1 l deionized water, components were dissolved in a hot water bath and the pH was adjusted to 7.2 ± 0.5 using 1 M NaOH. Finally, medium was autoclaved for 15 min at 121 °C and stored at room temperature in the dark for a maximum period of 6 months.

Modified Scholtens' Agar (MSA)

Composition: peptone: 10 g, yeast extract: 3 g, meat extract: 12 g, NaCl: 3 g, Na_2CO_3 solution (150 g/l): 5 ml, agar: 15 g, $MgCl_2$ solution (100 g $MgCl_2$ x 6 H_2O in 50 ml deionized water): 0.3 ml. After addition of 1 l deionized water, components were dissolved in a hot water bath and the pH was adjusted to 7.2 ± 0.5 using 1 M NaOH. The medium was autoclaved for 15 min at 121 °C, cooled to 48 °C in a water bath and 1.2 ml of a filter sterilized (0.2 µm) $CaCl_2$ solution (14.6 g $CaCl_2$ x 2 H_2O in 100 ml deionized water) were added per 200 ml of

medium. After mixing, MSA was poured into Petri dishes with 20 ml per plate and stored at 4 °C in the dark for a maximum period of 6 months.

Semi-solid Modified Scholtens' Agar (ssMSA)

Composition: peptone: 10 g, yeast extract: 3 g, meat extract: 12 g, NaCl: 3 g, Na_2CO_3 solution (150 g/l): 5 ml, agar: 7.5 g, $MgCl_2$ solution (100 g $MgCl_2$ x 6 H_2O in 50 ml deionized water): 0.3 ml. After addition of 1 l deionized water, components were dissolved in a hot water bath and the pH was adjusted to 7.2 $\pm$ 0.5 using 1 M NaOH. The medium was autoclaved for 15 min at 121 °C and stored at room temperature in the dark for a maximum period of 6 months. Prior use, ssMSA medium was dissolved in a hot water bath, cooled to 48 °C and 300 µl of a filter sterilized (0.2 µm) $CaCl_2$ solution (14.6 g $CaCl_2$ x 2 H_2O in 100 ml deionized water) were added per 50 ml of medium.

ATCC Medium 712

Composition of the basal medium: proteose peptone: 20 g, yeast extract: 1 g, $C_6H_5Na_3O_7$ x 2 H_2O: 1 g, 0.05 M $CaCl_2$ solution: 8 ml, 0.4 M $MgSO_4$ x 7 H_2O solution: 10 ml, 0.25 M KH_2PO_4 solution: 10 ml, 5 mM $Fe(NH_4)_2(SO_4)_2$ x 6 H_2O solution: 10 ml, 0.25 M Na_2HPO_4 x 7 H_2O solution: 10 ml. Deionized water was added to a final volume of 1 l. The pH was adjusted to 6.5 + 0.2 with 1 M NaOH and the medium was autoclaved for 15 min at 121 °C. Basal medium was stored at room temperature in the dark for a maximum period of 6 months. Prior to every use, a 2 M glucose solution was prepared and 500 µl of the glucose solution were added per 10 ml of basal medium. Finally, the complete medium was filter sterilized (0.2 µm).

Fetal bovine serum (FBS)

Fetal bovine serum (FBS, F0804, Sigma-Aldrich, St. Louis, USA) was thawed, portioned in sterile 50 ml centrifuge tubes and incubated for 30 min at 56 °C in a heating cabinet. Subsequently, FBS was stored at -20 °C until use.

Dulbecco's Modified Eagle's medium (DMEM) with fetal bovine serum (FBS)

500 ml Dulbecco's Modified Eagle's medium (DMEM, D6429, Sigma-Aldrich, St. Louis, USA) were mixed with 5 ml of 100 x antibiotic/antimycotic solution (A5955, Sigma-Aldrich, St. Louis, USA) under aseptic conditions. 10 % (v/v) FBS were added under aseptic conditions and the medium was stored at 4 °C for a maximum period of 1 month.

4. Methods

4.1 Cultivation of eukaryotic cell lines and microorganisms

4.1.1 Eukaryotic cell lines

Human lung carcinoma cells (A549) were grown in tissue culture flasks (T75 Standard, Sarstedt, Nürnbrecht, Germany) using DMEM supplemented with 10 % (v/v) FBS and 1 x antibiotic/antimycotic solution at 37 °C under 5 % CO_2 atmosphere. Cell growth was monitored microscopically and cells were subcultured when ~ 90 % confluence was reached. First, medium was removed and adherent cells were washed three times with 5 ml sterile Dulbecco's phosphate-buffered saline (DPBS, pH 7.1-7.5, D8537, Sigma-Aldrich, St. Louis, USA) before 3 ml pre-warmed trypsin-EDTA-solution (T4174, Sigma-Aldrich, St. Louis, USA) were added and cells were incubated for 2-5 minutes at 37 °C until detachment of cells was observed. Subsequently, 10 ml fresh medium (DMEM with 10 % (v/v) FBS and 1 x antibiotic/antimycotic solution) were added to inactivate trypsin-EDTA. Cells were centrifuged for 5 min at 1,000 x g, supernatant was removed and the pellet was resuspended in 5 ml fresh medium. Then, 1 ml of the cell suspension was added to a new tissue culture flask with fresh medium.

Murine macrophage cells (RAW 264.7) were grown in DMEM supplemented with 10 % (v/v) FBS and 1 x antibiotic/antimycotic solution at 37 °C under 5 % CO_2 atmosphere as described for A549 cells. For subculture, cells were carefully scraped off from the tissue culture flask bottom using a sterile cell scraper. Cells were centrifuged for 5 min at 1,000 x g, supernatant was removed and the pellet was resuspended in 5 ml fresh medium. Then, 1 ml of the cell suspension was added to a new tissue culture flask with fresh medium.

African rhesus monkey kidney cells (MA-104) were grown in DMEM supplemented with 10 % (v/v) FBS, 1 x antibiotic/antimycotic solution, 1 % (v/v) non-essential amino acids (M7145, Sigma-Aldrich Co., St. Louis,MO) and 2 mM L-glutamine (G7513, Sigma-Aldrich Co., St. Louis,MO) at 37 °C under 5 % CO_2 atmosphere as described for A549 cells.

For cryopreservation of all cell types, cells were suspended in DMEM with 20 % (v/v) FBS, 10 % (v/v) DMSO and 1x antibiotic/antimycotic solution, transferred to cryo vials and placed in a freezing container (Nalgene, Rochester, USA) at -70 °C to maintain a freezing rate of 1 °C/min. After one day, vials were transferred to liquid nitrogen and stored until used.

4.1.2 Cultivation of *Acanthamoeba castellanii*

Acanthamoeba castellanii ATCC® 30234™ were cultivated statically in ATCC® medium 712 in sterile tissue culture flasks (T25 Standard, Sarstedt, Nürnbrecht, Germany) at 25 °C. When the culture reached the stationary growth phase after 5-10 d, medium containing planktonic amoebae was removed and adherent amoebae were washed three times with 5 ml fresh medium. Subsequently, amoebae were covered with 1 ml medium and scraped off using a sterile cell scraper. Finally, 100 µl of the cell suspension were transferred to a new culture flask with fresh medium.

4.1.3 Preparation of virus stocks

In order to prepare virus stocks, MNV, HAdV and coliphage φX174 were propagated as follows: Murine noroviruses were propagated in murine macrophage (RAW 264.7) cells. Cells were grown as described before (4.1.1) and monitored microscopically until ~ 90 % confluence was reached. Then, medium was removed and DMEM supplemented with 2.5 % (v/v) FBS and 1 x antibiotic/antimycotic solution was added. 50 µl of a virus stock were added to the medium, the culture flask was gently agitated and incubated at 37 °C under 5 % CO_2 atmosphere until a morphological change of the cells and detachment of cells (cytopathic effect, CPE) was observed microscopically. Subsequently, cells were subjected to three freeze/thaw cycles (-20 °C/room temperature). Then, cultures were centrifuged for 5 min at 500 x g, the supernatant containing viruses was removed, aliquoted and stored at -70 °C until use.

Human adenoviruses were propagated in human lung carcinoma (A549) cells as described for murine noroviruses.

Coliphages φX174 were propagated in *E. coli* DSM 13124. First, 50 ml of MSB were inoculated with 500 µl of *E. coli* stock and incubated in a shaking water bath at 37 °C. The *E. coli* stock culture was previously prepared as described in DIN EN ISO 10705-2 (2000). After two hours of incubation, 400 µl of a coliphage φX174 stock suspension were added and incubation was continued for two more hours. Finally, the culture was centrifuged for 10 min at 11,000 x g. The supernatant was aliquoted and stored at -70 °C until use.

4.2 Microbiological methods

4.2.1 Total cell count

The total cell count was determined microscopically after staining with 4',6-diamidino-2-phenylindole (DAPI). Four milliliters of water or biofilm suspension were mixed with 1 ml of DAPI-solution (25 µg/ml in 2 % formaldehyde) and incubated for 20 min at room temperature in the dark. After incubation, samples were filtered through black polycarbonate filters (0.2 µm pore size, 30 mm diameter, Merck Millipore, Darmstadt, Germany) and filters were stored at 4 °C in the dark until analysis. Cells were counted using an epifluorescence microscope (AxioScope.A1, Carl Zeiss MicroImaging GmbH) with a counting grid (100 µm x 100 µm) at 1,000 x magnification (Objective: Zeiss N-Achroplan 100x/1.25 oil). A total number of 20 randomly selected fields of view were considered and counts between 10 and 100 cells were used for calculation. The total cell count was given as cells per milliliter of water (cells/ml) or gram (wet weight) of biofilm (cells/g).

4.2.2 Heterotrophic plate count

The number of culturable heterotrophic bacteria was determined by the spread plate method on R2A medium. Briefly, water or biofilm suspensions were serially diluted in sterile ultrapure water and 100 µl of diluted biofilm suspension was plated on R2A in triplicates and the plates were incubated at 20 °C for 7 d. After incubation, plates with 30 to 300 colonies were used for enumeration and the concentration was calculated as colony forming units per milliliter of water (CFU/ml) or gram (wet weight) of biofilm (CFU/g).

4.2.3 Enumeration of somatic coliphages

The number of infectious somatic coliphages was determined according to DIN EN ISO 10705-2 (2000). *Escherichia coli* DSM 13127 was grown in Modified Scholten's Broth (MSB) at 36 $\pm$ 2 °C, stock and working cultures were prepared as described in DIN EN ISO 10705-2 (2000). For enumeration of coliphages, samples were serially diluted in sterile DPBS and 500 µl of either diluted or undiluted suspension were mixed with 1 ml of a log-phase culture of *E. coli* and 2.5 ml ssMSA medium. Subsequently, the mixture was poured onto MSA plates, the plates were incubated for 18 $\pm$ 2 h at 36 °C and plaques were counted. Results were expressed as plaque forming units per milliliter of water (PFU/ml) or gram (wet weight) of sediment or biofilm (PFU/g), respectively. For each assay, negative and positive

controls were included to verify growth of *E. coli* (*E. coli* + DPBS instead of sample) and the detection of coliphages (*E. coli* + ~50 PFU of coliphage φX174).

4.2.4 Enumeration of *E. coli*

Enumeration of *E. coli* in samples of water, epilithic biofilms and sediment biofilms was performed in a most-probable-number (MPN) format using the Colilert-18/Quanti-Tray/2000 system according to the manufacturer's instructions (IDEXX GmbH, Germany) and DIN EN ISO 9308-2:2014-06 (2014). Sample dilutions were carried out in filter-sterilized (0.2 µm pore size cellulose acetate membrane filter) surface water from the corresponding sampling point. Water samples were diluted 1:10 for analysis, for epilithic biofilms and sediments, 1 g (wet weight) of epilithic biofilm or sediment was filled up to 100 ml with filtered surface water in a sterile glass beaker. Samples were then sonicated in an ultrasonic bath (Sonorex RK 103 H, Bandelin electronic, Berlin, Germany) for 20 min. Ice was added to the bath to ensure that temperature did not exceed 20 °C during the treatment. Homogenized samples were diluted 1:10 in sterile filtered surface water and subsequently, 100 ml of the diluted samples were used for analysis in a Quanti-Tray/2000. After 18 h of incubation at 35 $\pm$ 0.5 °C, wells with yellow color and blue fluorescence under UV-light were considered positive for *E. coli* and results were obtained from the table of the manufacturer. *E. coli* concentrations were expressed as MPN per l of water or kg (wet weight) of epilithic biofilm or sediment, respectively. The limit of quantification was calculated as 0.1 MPN per ml of water and 1 MPN per g of biofilm or sediment.

4.2.5 Isolation of viruses from surface water

For the isolation of enteric viruses from surface water, 10 l of water were concentrated. First, all samples were spiked with 100 µl of a stock suspension of murine norovirus (approximately 3.0×10^2 genome equivalents (gen eq.) per sample) to determine recovery rates. The samples were concentrated according to the modified VIRADEL protocol described by Hamza et al. (2009). In brief, the pH of water samples was adjusted to 3.5 with 1 M HCl and 100 ml of a 5 M $MgCl_2$ solution was added to a final concentration of 0.05 M. After filtration through a type HA negatively charged membrane (0.45 µm pore size, 142 mm diameter, Millipore, Bedford, USA) in a pressure pump system, the membrane was washed once (filtration of 200 ml of 0.5 M H_2SO_4, pH 3.5). Subsequently, viruses were eluted using 70 ml elution

buffer (0.05 M KH_2PO_4, 1 M NaCl, 0.1% (v/v) Triton X-100, pH 9.2), the eluate was neutralized with 1 M HCl. Thereafter, 12.5 % (w/v) PEG-6000 (Merck, Darmstadt, Germany) and 2.5 % (w/v) NaCl (Bernd Kraft GmbH, Duisburg, Germany) were added in solid form and samples were stirred overnight at 4 °C. The next day, samples were centrifuged at 17,000 x g for 15 min at 4 °C, the pellet was resuspended in 3 ml DPBS and the suspension was stored at -20 °C for further analysis.

4.2.6 Isolation of viruses from surface water biofilms and sediments

In order to quantify viruses in biofilms and sediments, a method for separation of viruses from the biofilm and sediment matrix had to be found. Recovery experiments were performed using sediment from the River Ruhr spiked with coliphage φX174 and HAdV to test five different protocols in triplicates based on a procedure published by Helmi et al. (2008). Spiking was performed by addition of 20 µl from each virus stock (~10^7 gen.eq.) into the mixture of sediment and elution buffer. The five elution protocols were as follows: **1:** 10 g (wet weight) of sediment were mixed with 30 ml elution buffer A (0.05 M KH_2PO_4, 1 M NaCl, 0.1 % (v/v) Triton-X-100, pH 9.4) based on Hamza et al. (2009) and subsequently sonicated for 20 min in an ultrasonic bath (Sonorex RK 103 H, Bandelin electronic, Berlin, Germany). During sonication, ice was added to ensure that the temperature did not exceed 20 °C. After sonication, samples were centrifuged (3,000 x g, 15 min, 4 °C) and the supernatant was subjected to a PEG concentration step as described before (4.2.5). **2:** The same protocol as for 1, but elution buffer B (1 % (w/v) beef extract, 0.375 % (w/v) glycine, pH 9.4) according to Helmi et al. (2008) was used. **3:** The same protocol as for 1 and 2, but elution buffer C (0.1 % (w/v) skim milk, 0.375 % (w/v) glycine, pH 9.4) according to Botzenhart and Hock (2002) was used. **4:** Protocol and elution buffer was used as for 2, but instead of the PEG concentration step, the following precipitation step was performed: adjustment of pH to 3.5 with 1 M HCl, stirring for 30 min on a magnetic stirrer, centrifugation at 17,000 x g for 15 min at 4 °C to recover viruses. **5:** The same protocol as for 4, but elution buffer C (0.1 % (w/v) skim milk, 0.375 % (w/v) glycine, pH 9.4) according to Botzenhart and Hock (2002) was used.

Recovery rates were calculated by dividing the concentration measured after virus elution by the concentration added to the samples. Later, protocol 2 was used for elution of viruses from

all biofilm and sediment samples. Due to the smaller sample volume, only 5 g (wet weight) of epilithic biofilm was used instead of 10 g (as for sediment samples).

4.2.7 Isolation of viruses from water and biofilms from drinking water systems for animals

For the concentration of animal viruses from water and biofilm samples from drinking water systems for animals, the sample processing only included a PEG concentration step as described before (4.2.5). To test the efficacy of this step, coliphage φX174, HAdV and MNV (~ 10^8 gen.eq./ml) were added to 45 ml of deionized water, and subsequently concentrated by the PEG concentration step. In addition, positive controls were prepared by adding the same number of viruses to 3 ml DPBS. All samples were analyzed in parallel via qPCR (4.3.1) and plaque assay (4.2.3) The recovery was calculated by dividing the concentration of each viruses in the concentrated sample by the concentration in the positive control.

4.2.8 Virus quantification using cell culture

In order to quantify infectious enteric viruses, eukaryotic cells were seeded in 48-well plates and subsequently exposed to the water and biofilm samples. For detection of infectious HAdV, A549 cells were grown in DMEM supplemented with 10 % (v/v) FBS and 1 x antibiotic/antimycotic solution at 37 °C under 5 % CO_2 atmosphere. MNV were quantified using RAW 264.7 cells grown with the same medium and growth conditions as for A549 cells. RoV A and EV were quantified using MA-104 cells grown under the same growth conditions, but the medium was additionally supplemented with 1% (v/v) non-essential amino acids and 2 mM L-glutamine.

Drinking water and biofilm samples were diluted 1:10 in DMEM without FBS, homogenized by vortexing and subsequently centrifuged (3,000 x g, 5 min) to remove bacteria that could interfere with cell growth. The supernatant was serially diluted in DMEM without FBS supplemented with 1 x antibiotic/antimycotic solution. For isolated viruses from river water (4.2.5), samples were serially diluted in DMEM without FBS supplemented with 1 x antibiotic/antimycotic solution without the centrifugation step.

When the cells reached ~ 90 % confluence (which was observed microscopically under 100x magnification), the respective medium was aspirated and adherent cells were washed three

times using 300 µl sterile DPBS. Then, 50 µl of the respective sample dilution was added to 6 wells of the 48-well plate. After 90 minutes of incubation at 37 °C under 5 % CO_2, 300 µl of maintenance medium (DMEM with 2.5 % (v/v) FBS and 1x antibiotic/antimycotic solution and additional 1 % (v/v) non-essential amino acids and 2 mM L-glutamine for MA-104 cells) were added to each well without removing the sample inoculum and cells were further incubated at 37 °C under 5 % CO_2 for 7 days. During this period, cells were monitored microscopically (100x magnification) for morphological changes and detachment of the cells (cytopathic effect, CPE). For each plate, six negative controls with addition of 50 µl DMEM instead of sample were included. The tissue culture infective dose ($TCID_{50}$) was calculated according to Reed and Muench (1938), results were expressed as $TCID_{50}$ per ml of water or g (wet weight) of biofilm. In order to determine the effect of water and drinking water biofilms on A549 cells and RAW 264.7 cells, water and 14 d old drinking water biofilms without addition of the target viruses were analyzed via cell culture as described above. Figure 5 shows representative examples of microscopic images for A549 cells and RAW 264.7 cells.

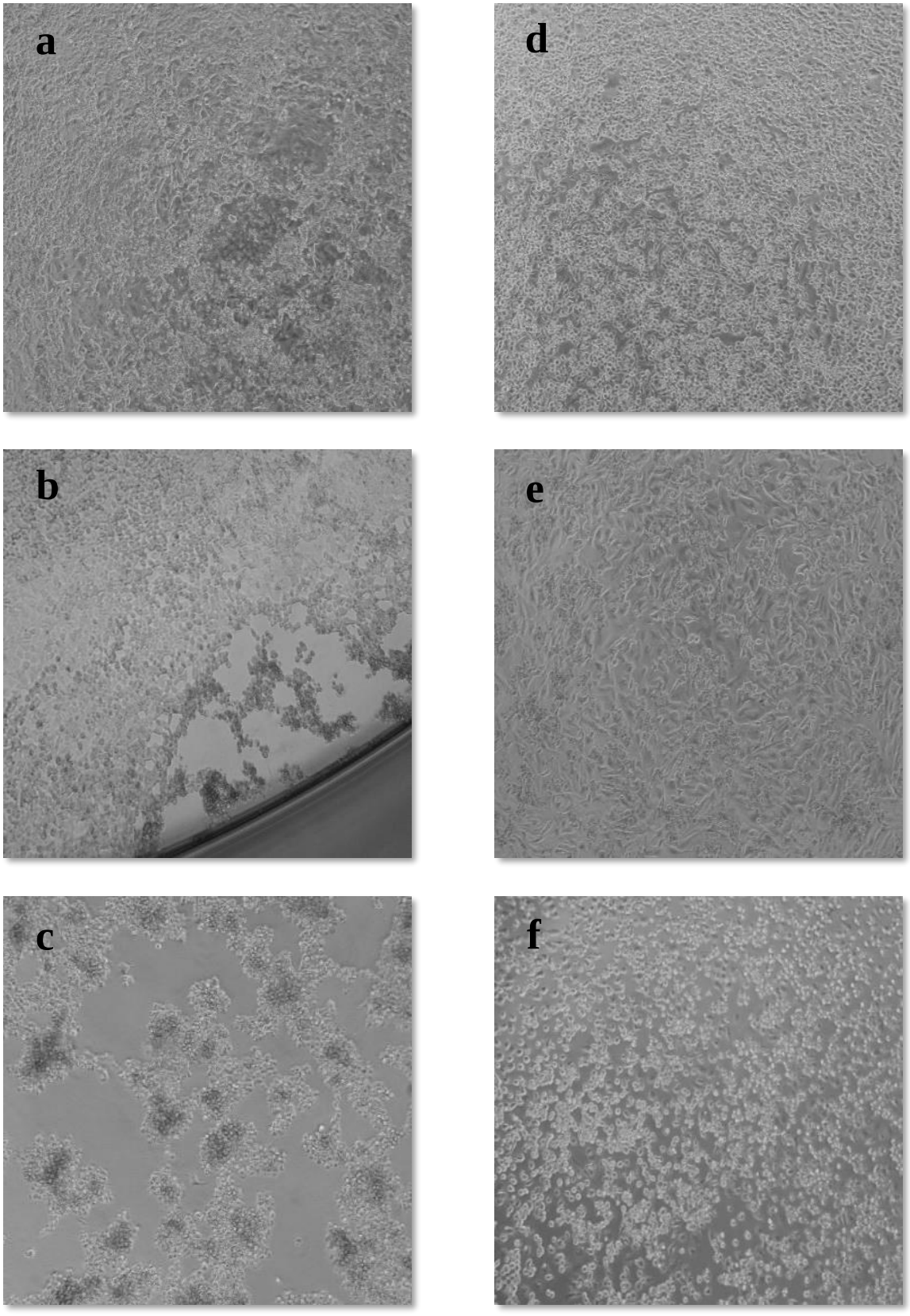

Figure 5: Representative light microscopic images (100x magnification, phase contrast) of viral cell cultures. ***a****: A549 cells, uninfected control;* ***b****: A549 cells, early CPE;* ***c****: A549 cells, late CPE;* ***d****: RAW 264.7 cells, uninfected control;* ***e****: RAW 264.7 cells, early CPE;* ***f****: RAW 264.7 cells, late CPE*

4.3 Molecular biology methods

4.3.1 Quantitative real-time PCR

Nucleic acids were extracted from 200 µl of virus suspensions (4.2.5, 4.2.6, 4.2.7) using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, extracted DNA was stored at -20 °C until analysis via qPCR. For the detection of RNA viruses, extracted samples were subjected to a reverse transcription step using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. In brief, 10 µl per sample was mixed with 10 µl of the RT-mastermix containing buffer, random primers, dNTPs, MultiScribe™ Reverse Transcriptase and RNase Inhibitor. Temperature settings for the cDNA synthesis were as follows: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min. Subsequently, cDNA was stored at -20 °C until further analysis via qPCR.

To quantify viruses in the samples, extracted DNA or cDNA were analyzed using qPCR with a Rotorgene 3000 cycler system (Corbett Research, Sydney, Australia) for river water samples and a CFX96 Touch™ Real Time PCR Detection System (Biorad, Hercules, USA) for all other samples with the QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The temperature settings of the qPCR detection for the different viruses are listed in Table 7. Primers, probes primer and their final concentrations in the PCR reaction are listed in Table 8.

Table 7: Temperature protocols for the detection of viruses using qPCR.

Virus	Step	Temperature and duration
Human adenovirus (HAdV)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 15 s
	Annealing and elongation	60 °C for 60 s
Human enterovirus (EV)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 15 s
	Annealing and elongation	60 °C for 60 s
Rotavirus group A (RoV)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 15 s
	Annealing	56 °C for 30 s
	Elongation	72 °C for 30 s
		74 °C for 5 s
Human norovirus genogroup GII (NoV GII)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 15 s
	Annealing and elongation	63 °C for 60 s
Murine norovirus (MNV)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 15 s
	Annealing and elongation	60 °C for 60 s
Coliphage φX174 (φX174)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 15 s
	Annealing and elongation	60 °C for 60 s
Porcine adenovirus (PAdV)	Initial denaturation	95 °C for 10 min
	Denaturation	95 °C for 10 s
	Annealing and elongation	55 °C for 20 s
		60 °C for 20 s

Table 7: Continued.

Virus	Step	Temperature and duration
Hepatitis E virus (HEV)	Initial denaturation	95 °C for 15 min
	Denaturation	95 °C for 10 s
	Annealing	55 °C for 20 s
	Elongation	72 °C for 15 s
Porcine reproductive and respiratory syndrome virus (PRRSV)	Initial denaturation	95 °C for 2 min
	Denaturation	95 °C for 15 s
	Annealing	56 °C for 20 s
	Elongation	72 °C for 30 s
Porcine circovirus type 2 (PCV2)	Initial denaturation	95 °C for 10 min
		50 °C for 2 min
	Denaturation	95 °C for 15 s
	Annealing and elongation	60 °C for 60 s

Absolute quantification of genome equivalents (gen.eq.) for each of the viruses in the samples was performed by comparing cycle threshold (ct) values to those of DNA/RNA standards which were included in every qPCR run. DNA and RNA standards for HAdV, MNV, EV, RoV, NoV GII and coliphage φX174 were kindly provided by Dr. Ibrahim Ahmed Hamza Ewess. The preparation was done as previously described by Hamza et al. (2009). For animal pathogenic viruses, no standards were available, thus DNA-standards for PAdV and HEV were prepared from customized oligonucleotides (Integrated DNA Technologies, Inc.) based on the amplicon sequences of PAdV and HEV (8.1). After determination of the DNA concentration using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, serial dilution of DNA was performed in PCR grade water. All qPCR standards were aliquoted and stored at -70 °C until use. All samples and standards were analyzed in duplicates. Negative controls without template DNA were also included in each run.

Table 8: Primers, probes and their respective final concentration in a PCR reaction used in this study. Hydrolysis probes were labelled with FAM (6-carboxycfluorescein) at 5' and BHQ1 (Black Hole Quencher) at 3'.

Primer/Probe	c [nM]	Target gene	Amplicon size [bp]	Sequence (5'-3')	Reference
HAdV fwd	125	Hexon	132	GCCACGGTGGGGTTTCTAAACTT	(Hamza et al., 2014; Heim et al., 2003)
HAdV rev	125			GCCCCAGTGGTCTTACATGCACATC	
HAdV_probe	100			FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	
φX174 fwd	125	P08	73	CATCCCGTCAACATTCAAACG	(Modified after Myers et al. 2009)
φX174 rev	125			CGCCATTAATAATGTTTTCCGTAA	
φX174_probe	100			FAM-TCTCATCATGGAAGGCGCTGAA-BHQ1	
MNV fwd	125	VP1	92	AGAGGAATCTATGCGCCTGG	(Tajima et al., 2013)
MNV rev	125			GAAGGCGGCCAGAGACCAC	
MNV_probe	100			FAM-GGGCTGTTTGTGCGGAGTGGCG-BHQ1	
NoV GII fwd	125	ORF1–ORF2 junction	89	CAAGAGTCAATGTTTAGGTGGATGAG	(Jothikumar et al., 2005; Kageyama et al., 2003)
NoV GII rev	125			TCGACGCCATCTTCATTCACA	
NoV II_probe	100			FAM-AGATTGCGATCGCCCTCCCA-BHQ1	
EV fwd	125	5' UTR	196	CCTCCGGCCCCTGAATG	(Hamza et al., 2009)
EV rev	125			ACCGGATGGCCAATCCAA	
EV_probe	100			FAM-ACGGACACCCAAAGTAGTCGGTTCCG-BHQ1	

Table 8: Continued.

Primer/Probe	c [nM]	Target gene	Amplicon size [bp]	Sequence (5'-3')	Reference
RoV fwd	125	VP6	128	ATGGATGTCCTGTACTCCTTGTCAAAA	(Modified after Hamza et al. 2014)
RoV rev	125			TTCCTCCAGTTTGRAASTCATTTCC	
RoV_probe	100			FAM-ATAATGTGCCTTCGACAAT-BHQ1	
PAdV_fwd	900	Hexon	68	AACGGCCGCTACTGCAAG	(Hundesa et al., 2009)
PAdV_rev	900			AGCAGCAGGCTCTTGAGG	
PAdV_probe	225			FAM-CACATCCAGGTGCCGC-BHQ1	
HEV_fwd	250	ORF3	69	GGTGGTTTCTGGGGTGAC	(Jothikumar et al., 2006)
HEV_rev	250			AGGGGTTGGTTGGATGAA	
HEV_probe	100			FAM-TGATTCTCAGCCCTTCGC-BHQ1	
PRRSV_fwd	800	Nucleocapsid protein	77	GCACCACCTCACCCRRAC	(Wernike et al., 2012)
PRRSV_rev	800			CAGTTCCTGCRCCYTGAT	
PRRSV_probe	250			FAM-CCTCTGYYTGCAATCGATCCAGAC-BHQ1	
PCV2_fwd	900	Putative capsid protein	99	CCAGGAGGGCGTTGTGACT	(Olvera et al., 2004)
PCV2_rev	900			CGCTACCGTTGGAGAAGGAA	
PCV_ probe	150			FAM-TTTGCAGACCCGGAAACCACATACTGGA-BHQ1	

4.3.2 Agarose gel electrophoresis

To verify the size of PCR products, agarose gel electrophoresis was performed. Rotiphorese® 10x TBE buffer was diluted 1:10 in ultrapure water and served as running buffer. 1 g of agarose was added to 50 ml 1x TBE buffer and dissolved by heating in a microwave. After mixing with 0.5 µl of SYBR® Safe DNA Gel stain, the gel was poured and solidified in the dark. For each sample, 10 µl of PCR product were mixed with 2 µl of loading dye, and 10 µl of this mixture were added per lane. As a size control, a DNA marker (GeneRuler 1 kb DNA Ladder SM0311, Thermo Fisher Scientific) was used for each run. The gel was run in a gel electrophoresis unit (HE33, Amersham with Power Pack P25, Biometra) at 95 V with 70 mA for 30 – 60 min in the dark and bands were visualized using a Gel Documentation System (Universal Hood 2, Bio-Rad).

4.4 Chemical methods

4.4.1 Determination of magnesium and calcium in water

In order to determine the concentrations of magnesium ions and calcium ions in the drinking water, samples were analyzed by ion chromatography. Prior to analysis, water samples were centrifuged for 10 min at 13,000 x g. The supernatant was diluted 1:10 in ultrapure water and measured in triplicates. For analysis, a Dionex Aquion Ionchromatography System (Thermo Scientific) was used. The system was equipped with a Dionex IonPac™ CG12A (2 x 50 mm) guard column (Thermo Scientific), a Dionex IonPac™ CS12A (2 x 250 mm) analytical column (Thermo Scientific), a Dionex CERS 500 suppressor (Thermo Scientific) and a DS6 conductivity detector (Thermo Scientific). As eluent, 0.02 M methanesulfonic acid in ultrapure water was used with a flow rate of 0.25 ml/min (isocratically). Quantification was achieved by comparing samples with cation standards, ultrapure water was used as negative control in each run. Data acquisition was performed using the Software Chromeleon Version 7.2 SR5 (Thermo Scientific).

4.5 Field studies

4.5.1 Detection of viruses in river water, sediments and epilithic biofilms

In the period from July to September 2015, 24 samples each from water, epilithic biofilms and sediments were collected weekly at three different sampling sites along the River Ruhr including a barrier lake (Lake Baldeney) in the City of Essen, a densely populated metropolitan area in North Rhine Westphalia, Germany (Figure 6; SP1±51°26'30.4"N 7°04'09.4"E, SP2±51°24'22.0"N 7°01'11.2"E, SP3±51°22'51.5"N 6°59'51.1"E). Sampling sites were chosen based on a previous study (Strathmann et al., 2016). The sampling points SP1 (Schwimmverein Steele) and SP3 (Löwental) were located upstream and downstream of Lake Baldeney, while SP2 (Seaside Beach) was located at Lake Baldeney. For each sampling site, 11 liters of water, a sample of river sediment, and a sample of epilithic biofilm were collected. For sediments, a sample from the top layer was taken with a sterile metal spoon and transferred to a sterile conical tube. Macroscopically, sediments mainly consisted of sand and gravel, coarse fragments such as stones and wood bigger than approximately 1 cm in diameter were manually removed from the samples. Epilithic biofilm was scraped off from stones using a sterile rubber spatula and transferred into sterile conical tubes at the sampling site. The collection of samples was approved by the German Federal Ministry of Education and Research (BMBF project number 02WRS1283A to J) and did not involve endangered or protected species. Water temperature was measured at the sampling site.

The samples were transported into the laboratory in less than two hours under cooling and sample processing was conducted within the same day. Data for daily precipitation at the weather station in Essen-Bredeney were obtained online (Deutscher Wetterdienst, 2017). Additionally, average daily flow rate data from a site approximately 100 m upstream of SP3 were obtained online (Ruhrverband, 2016).

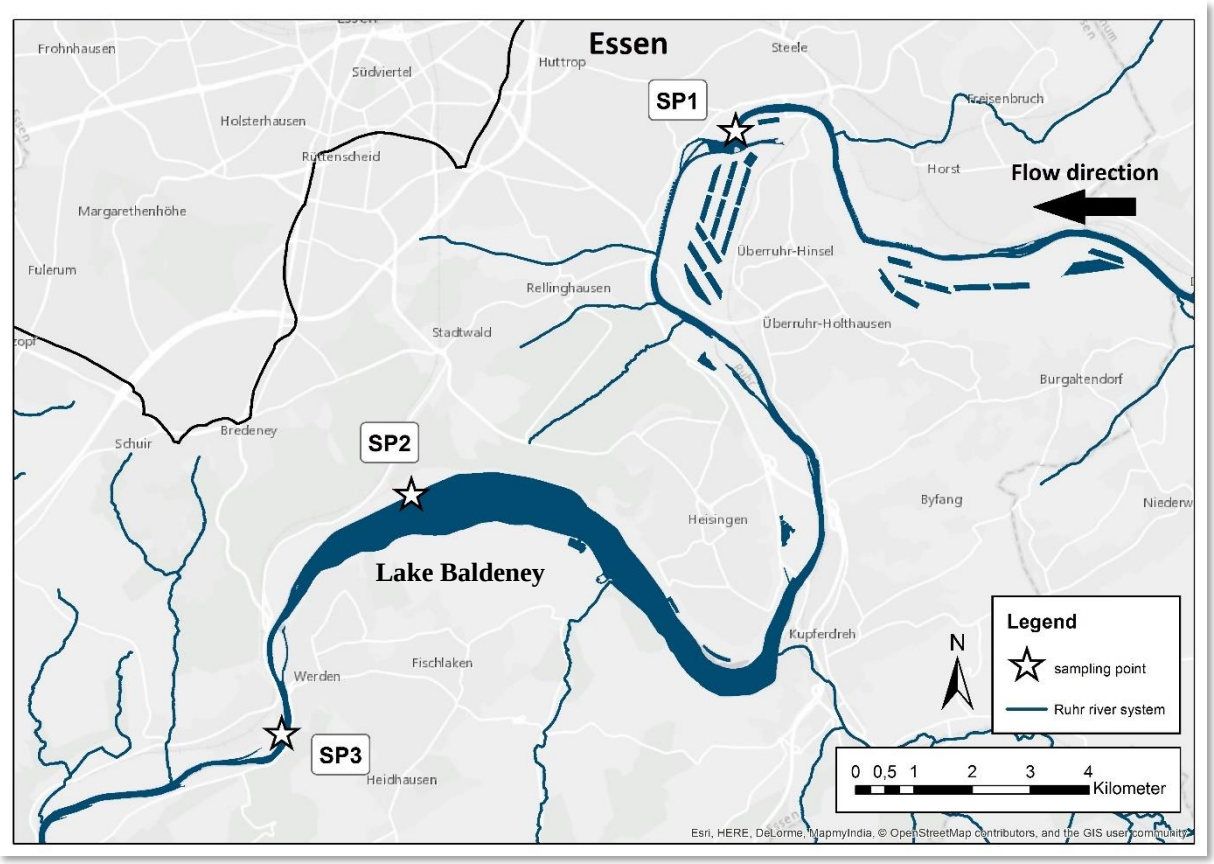

Figure 6: Sampling sites along the river Ruhr in Essen, Germany during the field study (Mackowiak et al., 2018).

4.5.2 Viruses in biofilms of drinking water systems for animals

In this field study, the occurrence of somatic coliphages and selected animal pathogenic viruses (PAdV, HEV, PRRSV, PCV2) in drinking water systems for animals was assessed. Water and biofilm samples used in this study were available from another research project (project title: Optimierung von Tränkwasser-Systemen für Schweine und Geflügel unter besonderer Berücksichtigung der Rolle mikrobieller Biofilme; funding: German Federal Ministry for Economic Affairs and Energy; project partners: IWW Water Centre (Mülheim an der Ruhr), University of Veterinary Medicine Hannover, Foundation (TiHo Hannover) - Field Station for Epidemiology, University of Duisburg-Essen – Biofilm Centre).

For this work, a total number of 84 samples, including 25 water samples and 59 biofilm samples were used, which originated from five different piglet breeding farms in Germany. The water samples included 9 samples from the inflowing water and 16 samples from the water distribution systems in the piglet breeding farms. The concentration of somatic coliphages was determined according to DIN EN ISO 10705-2 as described before (4.3.3).

For the detection of animal pathogenic viruses, water and biofilm suspensions were transferred to sterile 100 ml Schott-bottles and filled up with distilled water to a final volume of 45 ml. Subsequently, samples were processed as described above (4.3.7). In case of water samples, results were expressed as PFU or gen.eq. per ml of water. Because the wet weight of sampled biofilms was not determined, results for biofilms were expressed as gen.eq./cm^2 of sampled pipe surface. Negative controls (45 ml distilled water) were included for each set of samples.

4.6 Laboratory experiments

4.6.1 Interaction of enteric viruses and somatic coliphages with drinking water biofilms

Drinking water biofilms were grown on ethylene propylene diene monomer coupons (EPDM 65 Shore; Schmitztechnik GmbH, Germany) in stainless steel reactors (Figure 7a), the flow rate of 50 ml/min was maintained constant with a peristaltic pump.

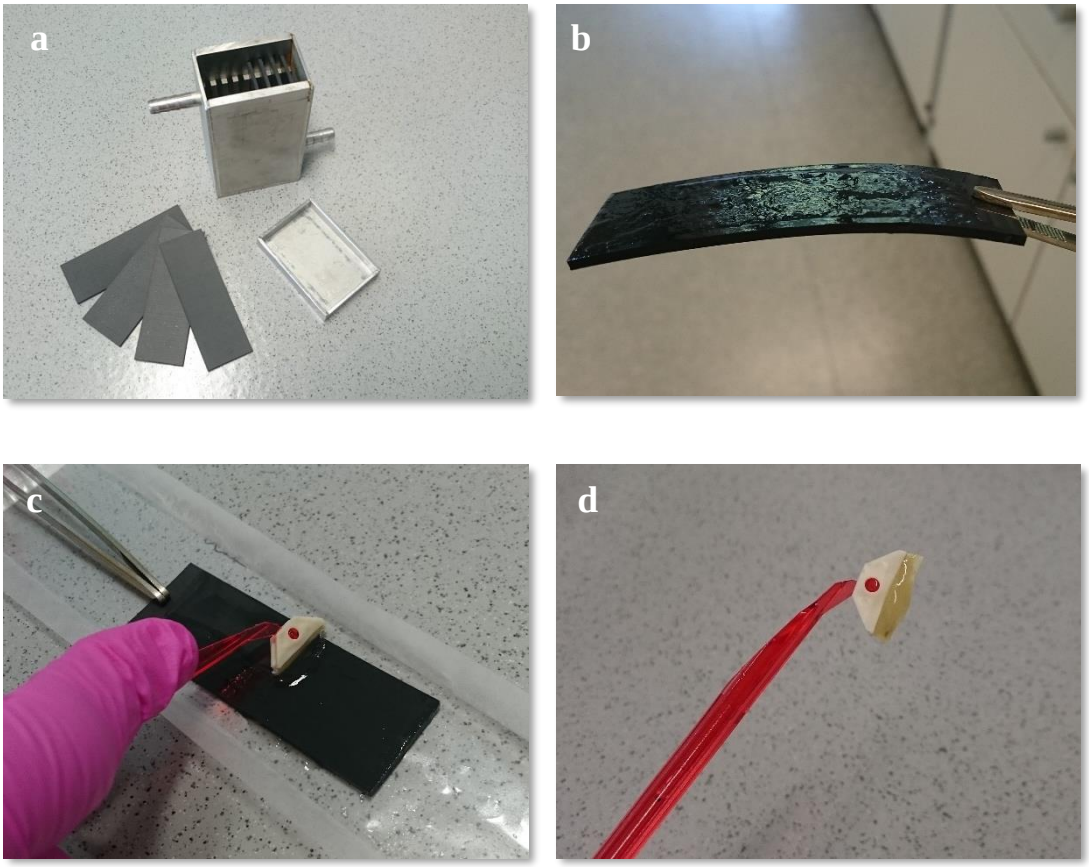

Figure 7: ***a*** *Stainless steel reactor with EPDM coupons* ***b*** *21d old drinking water biofilm on EPDM* ***c*** *Sampling of drinking water biofilm with a cell scaper* ***d*** *21d old drinking water biofilm collected on a cell scaper.*

In order to prevent retrograde contamination, the system was separated from the drinking water network by an open overflow (Figure 8). The water used in the experiments was drinking water which was stored intermediately in the building of the laboratory. Before connecting, reactors, EPDM coupons, tubes and tubing connectors were pasteurized for 10 min at 80 °C in a water bath. EPDM coupons were weighed and placed in the reactors using a sterile tweezer.

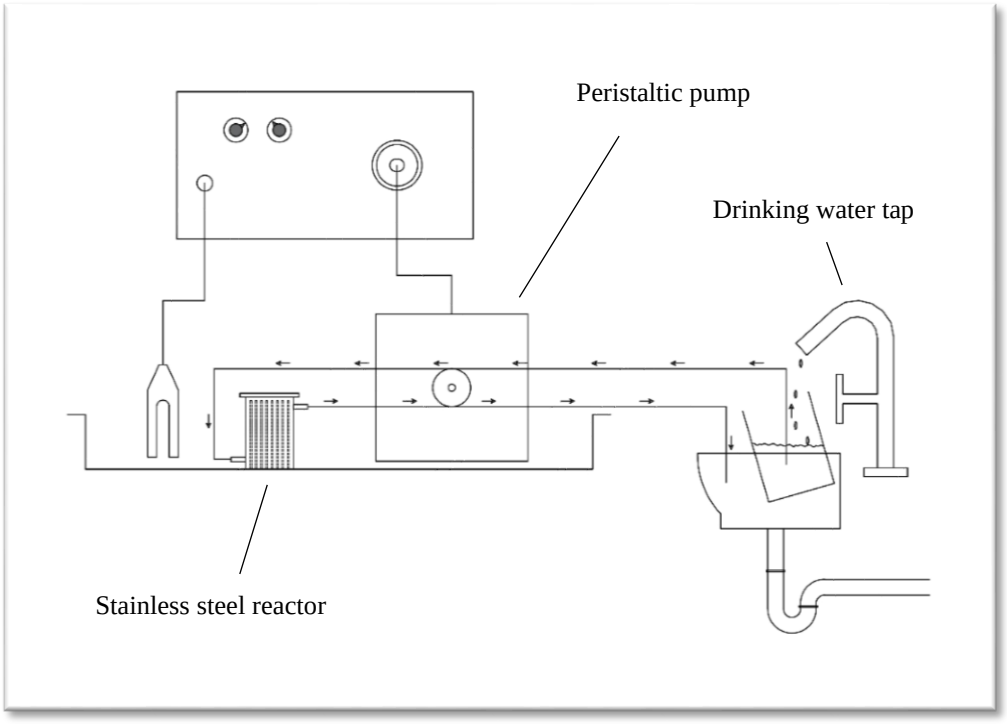

Figure 8: Scheme of the experimental setup for the growth of drinking water biofilms in a stainless steel reactor

During the period of biofilm growth, water temperature, pH, total and free chlorine were measured twice per day at the inlet of the reactors. Additionally, samples from the inflowing water were collected and stored at -20 °C for analysis by ion chromatography (4.4.1). After 14 d of biofilm growth, reactors were disconnected, viruses (~ 10^7 gen.eq./ml of each HAdV, MNV, coliphage φX174) were added to the water and reactors were operated in circular mode for 30 min to achieve homogeneous distribution within the system.

In order to assess the effect of water hardness on the interaction between biofilms and viruses, some reactors were run with hardened drinking water instead of drinking water for 1 h with a flow rate of 50 ml/min prior to addition of viruses. The hardened water was prepared just before use as follows: To reach a final concentration of approximately 4 mM Ca^{2+} (including already present Ca^{2+} in the drinking water), 3.14 g of $CaCO_3$ were added to 10 l of drinking water in a Schott bottle and 25 % HCl was added under continuous stirring until the carbonate was completely dissolved. Subsequently, 5 M NaOH was added until the pH was equal to the pH of the original drinking water again. Samples from the hardened drinking water were stored at -20 °C for analysis via ion chromatography (4.4.1). After the addition of viruses, reactors were either incubated under stagnant conditions or under flow conditions. For the flow conditions, inlet and outlet of the reactors were connected to a 1 l Schott bottle with 1 l of drinking water and flow rate was kept constant at 50 ml/min. At different time points,

samples were taken to quantify HAdV, MNV and coliphage φX174. Water samples were taken from the Schott bottle in case of flow condition experiments and directly from the reactors in case of stagnant conditions. To determine the reduction of virus concentrations in water, the reduction rate r was calculated for all viruses based on the logarithmic reduction at time points 1 d and 7 d (Equation 1):

$$r\ \left[\frac{log}{d}\right] = \frac{\log(\frac{N_{7d}}{N_0}) - \log(\frac{N_{1d}}{N_0})}{7d - 1d} \qquad (1)$$

With: r=reduction rate, N_{7d}=concentration after 7 d, N_{1d}=concentration after 1 d, N_0=concentration at the beginning of the incubation period

To quantify viruses in the drinking water biofilms, 4 EPDM coupons per reactor and sampling date were carefully removed from the reactor using sterile tweezers (Figure 7b) and weighed in a sterile glass beaker. Subsequently, the biofilm was scraped off from both sides with a sterile cell scraper (Figure 7c, d) and transferred into a sterile microcentrifuge tube. All biofilms from the same sampling date and reactor were collected in the same tube, which was weighed before and after the addition of biofilm in order to quantify the total wet mass of the biofilm. After that, sterile DPBS was added to the biofilm to reach a total volume of 800 µl. The biofilm suspension was homogenized by vortexing for 30 s and four aliquots of 200 µl each were prepared and stored at -20 °C until further analysis.

In order to determine the wet weight of (unwanted) biofilms that grew in tubing material used during the experiment, all tubes were weighed before and after the experiment.

4.6.2 Interaction between somatic coliphages and *E. coli* in monospecies biofilms

Escherichia coli monospecies biofilms were grown in 96-well polystyrene microtiter plates. A stock culture of *E. coli* DSM 13127 was prepared as described in DIN EN ISO 10705-2 (2000), thawed and diluted 1:100 in LB medium. Subsequently, 200 µl of this cell suspension were added per well of a 96-well microtiter plate and the plates were incubated at 20 °C or 36 °C for 48 h. After incubation, medium was removed and 200 µl fresh LB medium or sterile filtered (0.2 µm) surface water were added per well. Both types (LB and surface water) were previously inoculated with 10^3 gen.eq./ml or 10^6 gen.eq./ml of coliphage φX174. Medium or water without coliphages served as negative control. Incubation at 20 °C or 36 °C was continued for 4 h. In order to determine the phage titer, samples were taken at timepoints 0, 1 h, 4 h and stored at -20 °C until analysis via plaque assay (4.2.3). In order to quantify the extent of growth in microtiter plates at the end of the experiment, remaining biofilms were stained with crystal violet as follows: First, medium or water was removed, biofilms were washed three times with 200 µl deionized water per well and 150 µl of a 0.1 % (v/v) crystal violet solution was added. Plates were incubated for 20 min at room temperature in the dark, subsequently the crystal violet solution was aspired and stained biofilms were washed three times with 200 µl of deionized water. To dissolve the dye from the biofilms, 200 µl of 30 % acetic acid were added per well, incubated for 15 min at room temperature in the dark and the absorbance at 570 nm was measured in a microtiter plate reader (Infinite M200 PRO, Tecan Group AG). According to Merritt et al. (2011), the absorbance is proportional to the biofilm biomass.

4.6.3 Co-cultivation of *A. castellanii* and HAdV

In order to determine the potential uptake of HAdV by *A. castellanii*, a culture of *A. castellanii* ATCC 30234 as prepared as described before (4.1.2). Then, 50 µl of a stock suspension of HAdV (4.1.3) was added to the culture medium, the flask was gently agitated and incubated for 7 d at 25 °C. A 200 µl sample from the culture medium was taken at the beginning of the incubation period and stored at -20 °C until further analysis. At the end of the incubation period, the medium containing planktonic amoebae was removed and 200 µl samples were taken in triplicates and stored at -20 °C until further analysis. Adherent amoebae were washed three times with 2 ml of fresh medium and finally covered with 3.5 ml of fresh medium. After scraping off the amoebae with a sterile cell scraper, cells were transferred to three sterile 1.5 ml reaction tubes (each 1 ml cell suspension) and were washed

five times by centrifugation at 1,000 x g for 5 min and subsequent resuspension in 1 ml fresh medium. After the last centrifugation step, amoebae were resuspended in 200 µl of medium and stored at -20 °C until analysis. All samples were analyzed via qPCR (4.3.1).

5. Results

In this work, the interaction between viruses and aquatic biofilms was assessed using both field studies and laboratory studies. In two field studies, the fate of viruses in natural and technical aquatic systems was determined. In the first field study, viruses were quantified in river water, epilithic biofilms and sediments of an urban river in Essen, Germany. In a second field study, virus occurrence in water and biofilms from drinking water systems for animals was assessed. Laboratory studies were used to study the retention of selected model viruses in artificially grown drinking water biofilms under different conditions. Moreover, biological interactions between coliphage φX174 and *E. coli* monospecies biofilms were studied under laboratory conditions. In addition, the uptake of HAdV by *A. castellanii* in Co-cultivation was elucidated to determine the potential influence of amoebae on virus retention in biofilms.

5.1 Field studies

In the following part of this work, two field studies were conducted to determine the occurrence of viruses in natural and technical aquatic systems. First, a field study at the river Ruhr in Essen (Germany) was chosen to determine the distribution of somatic coliphages and human enteric viruses in a surface water environment. The Ruhr is an urban river, located in a densely populated area in Germany, and receives treated wastewaters and surface runoff. Moreover, it serves as raw water source for drinking water production and is used for recreational activities such as bathing. A second field study focused on the presence of coliphages and animal pathogenic viruses in drinking water systems for animals of piglet breeding farms in Germany, which were chosen as an example for a technical water system that is fecally polluted.

5.1.1 Detection of viruses in water, sediments and epilithic biofilms of the river Ruhr in Essen, Germany

In this field study, the distribution of somatic coliphages and human enteric viruses was determined in an urban surface water environment with anthropogenic pollution. Additionally, *E. coli* was included in this study because it commonly serves as bacterial indicator for fecal pollution and is a host for somatic coliphages. Microbial and physicochemical parameters were assessed in water, epilithic biofilms and sediments which were periodically sampled during this study.

5.1.1.1 Evaluation of virus elution efficiency

In order to recover viruses from epilithic biofilms and sediments, different methods for separation of matrix and viruses were tested prior to sampling (4.2.6). Three different elution buffers and two concentration protocols were tested after spiking ~10^7 gen.eq. HAdV and coliphage φX174 to 10 g (wet weight) of River Ruhr sediment. The results for virus recovery rates are shown in Figure 9.

Methods 1, 2 and 3 were more efficient for both viruses than methods 4 and 5. Thus it was decided to use the elution protocol 2 for elution of viruses from sediment and biofilm samples, which was based on sonication of samples in elution buffer (1 % (w/v) beef extract, 0.375 % (w/v) glycine, pH 9.4) followed by centrifugation and subsequent PEG concentration of the supernatant. The mean recovery rates of this method were 77 % for HAdV and 56 % for coliphage φX174, respectively (Figure 9). For water samples, the recovery rate for murine norovirus during sample treatment was between 50 % and 75 % (data not shown).

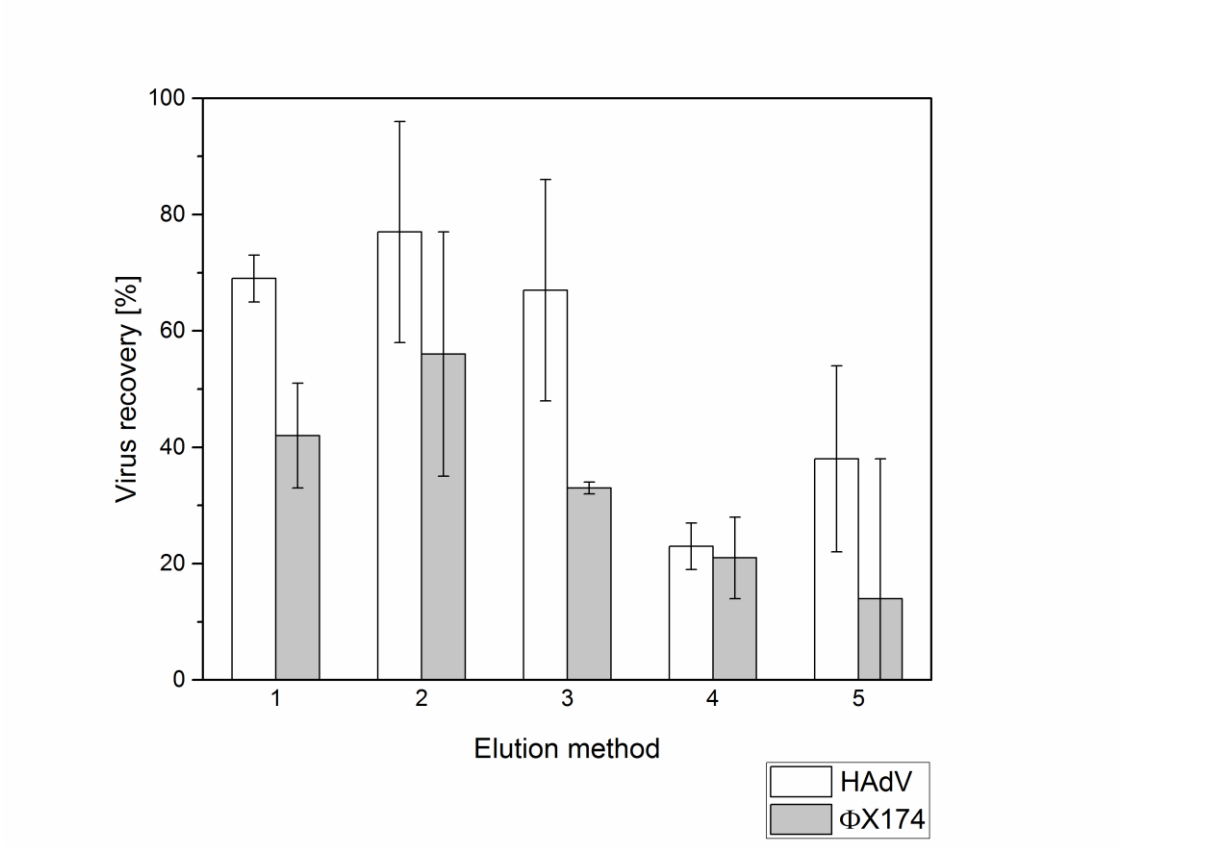

Figure 9: Mean recovery rates for different elution methods, tested after spiking of ~10^7 gen.eq. of HAdV and coliphage φX174 to 10 g (wet weight) to River Ruhr sediment. ***1****: Buffer A (KH_2PO_4) with subsequent PEG concentration,* ***2****: Buffer B (beef extract) with subsequent PEG concentration,* ***3****: Buffer C (skim milk) with subsequent PEG concentration,* ***4****: Buffer B (beef extract) with subsequent precipitation,* ***5****: Buffer C (skim milk) with subsequent precipitation. Experiments were performed in triplicates, error bars indicate the standard deviation. (Mackowiak et al., 2018)*

5.1.1.2 Characteristics of sampling sites

In the summer period of the year 2015, 24 samples each from water, epilithic biofilms and sediments of the River Ruhr at three sampling sites in the City of Essen, Germany (Figure 6) were collected. Sampling sites were located before (SP1), at (SP2) and after (SP3) a barrier lake (Lake Baldeney). The mean water temperature was 19.4 °C ± 2.3 °C (n = 24). No significant differences in water temperature between the sampling points in the period of sampling were observed (data not shown).

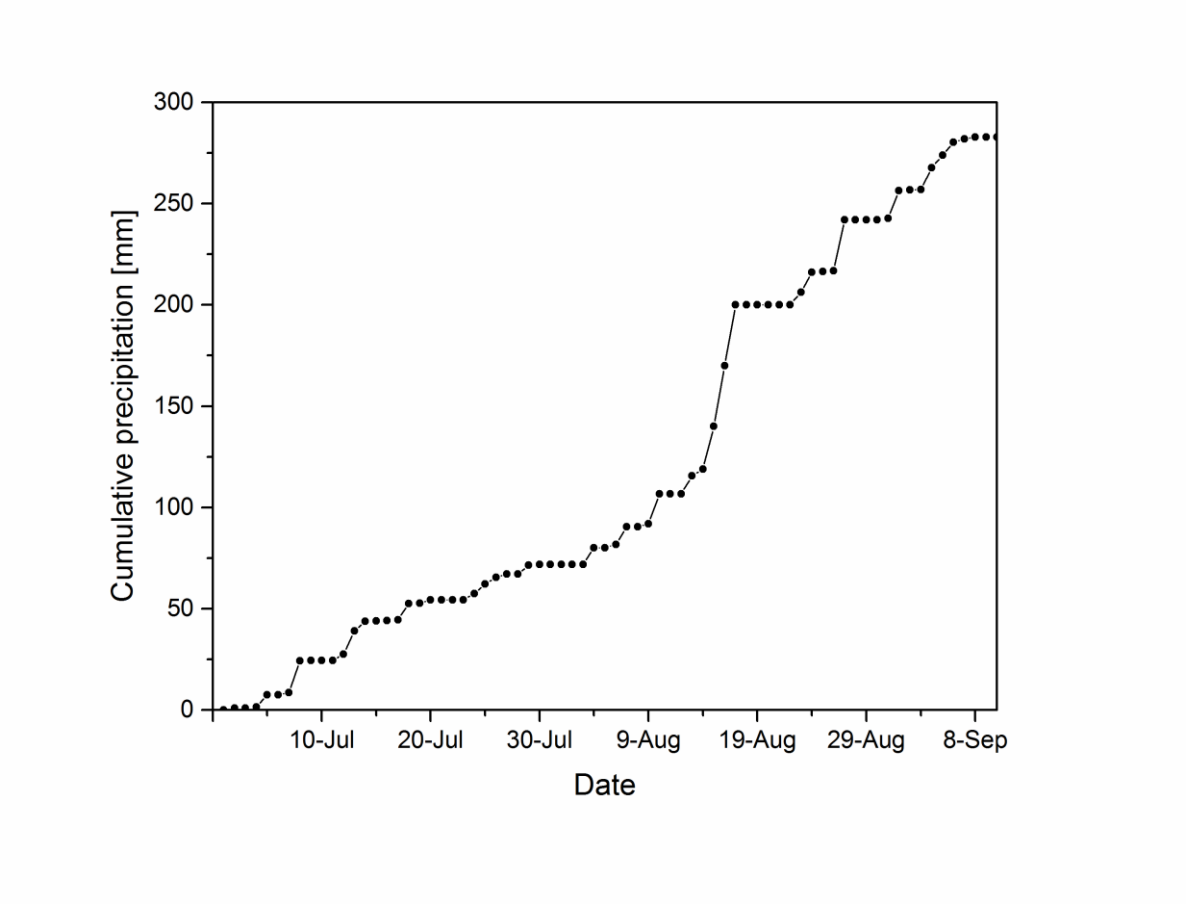

Figure 10: Cumulative precipitation at the weather station in Essen-Bredeney in the period from July 1 to September 10. Data were obtained online (Deutscher Wetterdienst, 2017). (Mackowiak et al., 2018)

The mean precipitation in the period from July 1 to September 10 was 3.9 ± 6.8 mm/d with a period of enhanced rainfall between August 15 and August 17 (daily precipitation of 30.0 mm on August 15 and 16). This rainfall caused a rapid increase in the cumulative precipitation in the sampling period (Figure 10). Prior to this study, the last period of enhanced rainfall had occurred in April 2015 (data not shown).

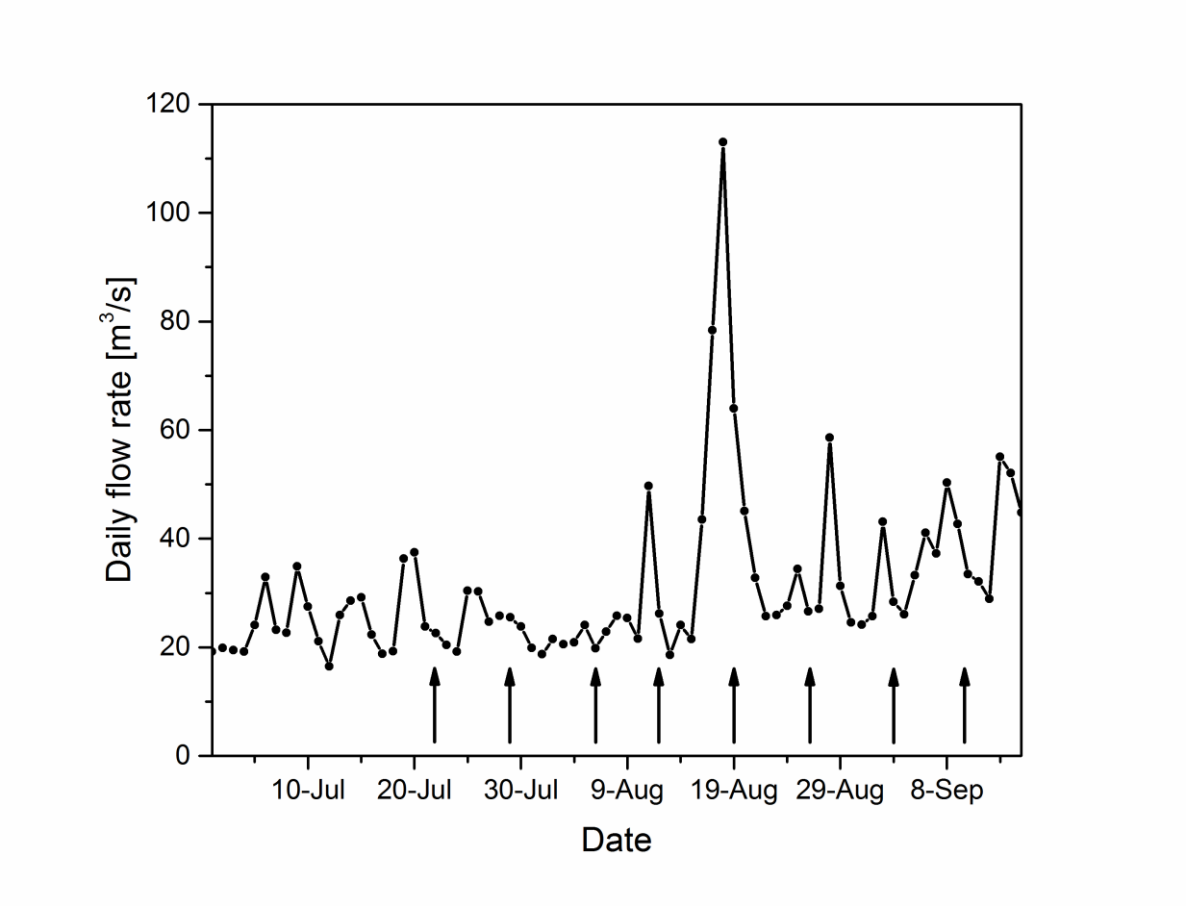

Figure 11: Daily flowrate in the period of sampling, measured 100 m upstream from sampling point SP3: "Löwental"; sampling dates are indicated by arrows. (Mackowiak et al., 2018)

Data for daily flow rates of the River Ruhr in approximately 100 m distance upstream from the sampling point "SP3: Löwental" were available online (Ruhrverband, 2016). The average daily flow rate in the period from July 22 to September 10 was 32 m^3/s with a minimum of 19 m^3/s and a maximum of 113 m^3/s (Figure 11). The maximum flow rate corresponded to the period of enhanced rainfall with a peak flow rate on August 18.

5.1.1.3 Distribution of *E. coli* and somatic coliphages in river water, biofilms and sediments

The concentration of the fecal indicator bacterium *E. coli* was determined culturally using the Colilert-18/Quanti-Tray/2000 system. All samples from water, epilithic biofilm and sediment tested for *E. coli* were positive (Table 9). For one water sample from the first sampling day (July 22), the concentration was above the range of quantification (>2419.6 MPN/100 ml). Two groups of samples were defined (before and after August 15) in order to compare the concentrations of *E. coli* before and after the period of enhanced rainfall. For water samples, the arithmetic mean *E. coli* concentration increased by approximately 2.5 log units in the period after August 15 compared to the period before August 15. Subsequently, the concentration decreased again slowly by approximately one log unit until September 10 (Figure 13a). In contrast to water samples, the concentration of *E. coli* in epilithic biofilms and sediments did not increase after the period of enhanced rainfall (Figure 13b). Over the whole sampling period, the mean concentration of *E. coli* in epilithic biofilms and sediments was approximately three orders of magnitude higher than in the flowing water before the period of enhanced rainfall. Moreover, the concentration in epilithic biofilms tended to be higher than in sediment samples.

Somatic coliphages were quantified using the plaque test according to DIN EN ISO 10705-2 (2000). In total, 22 of 24 water samples were positive for somatic coliphages, two samples showed results below the limit of detection (LOD = 2.5 PFU/l). All 24 samples of epilithic biofilms and 23/24 samples of sediments were positive for coliphages. In many cases, different plaque morphologies and sizes were observed (Figure 12).

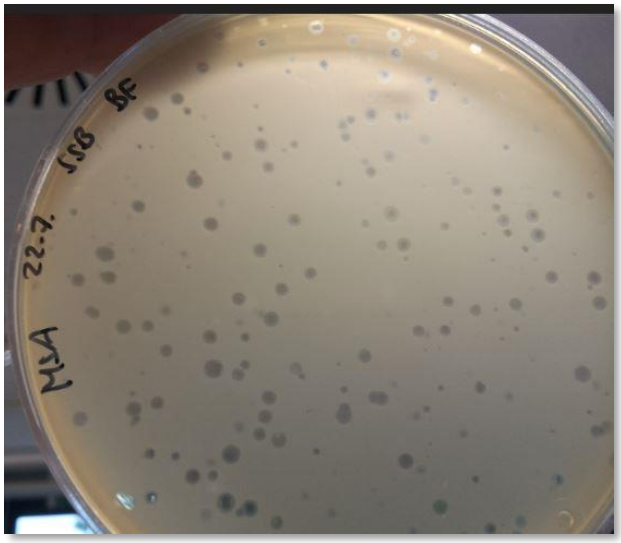

Figure 12: Plaques of different size and morphology in a lawn of E. coli DSM 13127 formed by somatic coliphages in a surface water biofilm sample from the River Ruhr.

Mean concentrations of coliphages were elevated in epilithic biofilms and sediments compared to the flowing water. After the period of enhanced rainfall, the mean concentration in water increased by one log unit (Figure 13c). In case of sediments, the mean concentration increased by more than one order of magnitude after the rainfall, whereas the mean concentration in epilithic biofilms before the rainfall remained almost constant after the period of enhanced rainfall (Figure 13d). The mean concentrations of *E. coli* and somatic coliphages in river water, epilithic biofilms and sediments before and after the period of enhanced rainfall are listed in Table 9.

Table 9: Percentage of positive samples and arithmetic mean concentrations of E. coli and somatic coliphages in river water, epilithic biofilms and sediments before and after the period of enhanced rainfall. (Mackowiak et al., 2018)

		Water		Epilithic biofilm		Sediment	
Organism	Sample group	% positive	Mean concentration	% positive	Mean concentration	% positive	Mean concentration
E. coli	Before rainfall	100 (10/10) [a]	$6.9 \times 10^3 \pm 3.7 \times 10^3$ MPN/l	100 (10/10) [a]	$4.4 \times 10^6 \pm 7.1 \times 10^6$ MPN/kg	100 (10/10) [a]	$3.4 \times 10^6 \pm 8.0 \times 10^6$ MPN/kg
	After rainfall	100 (12/12) [a]	$1.2 \times 10^6 \pm 1.3 \times 10^6$ MPN/l	100 (12/12) [a]	$5.4 \times 10^6 \pm 7.2 \times 10^6$ MPN/kg	100 (12/12) [a]	$3.0 \times 10^6 \pm 6.8 \times 10^6$ MPN/kg
Somatic coliphages	Before rainfall	92 (11/12) [a]	$5.8 \times 10^1 \pm 6.7 \times 10^1$ PFU/l	100 (12/12) [a]	$4.0 \times 10^5 \pm 1.2 \times 10^6$ PFU/kg	92 (11/12) [a]	$1.6 \times 10^5 \pm 2.8 \times 10^5$ PFU/kg
	After rainfall	92 (11/12) [a]	$5.2 \times 10^2 \pm 5.0 \times 10^2$ PFU/l	100 (12/12) [a]	$5.8 \times 10^5 \pm 8.5 \times 10^5$ PFU/kg	100 (12/12) [a]	$8.4 \times 10^6 \pm 2.5 \times 10^7$ PFU/kg

[a]In brackets, number of positive samples/total number of samples

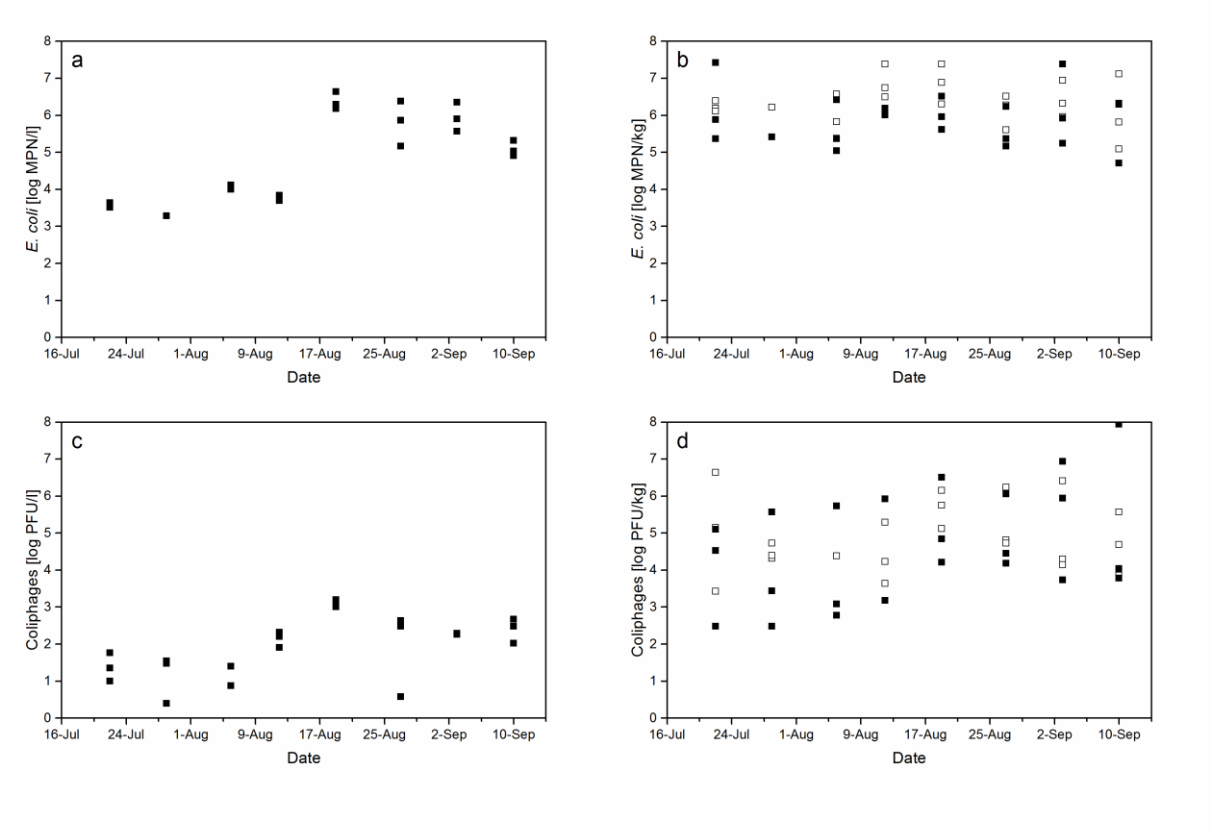

*Figure 13: Concentrations of E. coli in the period of sampling, given as most probable number (MPN) per l surface water, n=21 (**a**) and kg wet weight of biofilm, n=22 or sediment, n=22 (**b**). Concentrations of somatic coliphages in the period of sampling, given as plaque forming units (PFU) per l surface water, n=23, (**c**) and kg wet weight of biofilm, n=24 or sediment, n=23 (**d**). Black squares (b and d) correspond to sediment, white squares to epilithic biofilm. (Mackowiak et al., 2018)*

The mean ratio of coliphages to *E. coli* was higher in biofilms ($1.7 \times 10^{-1} \pm 3.8 \times 10^{-1}$) and sediments ($2.3 \times 10^{0} \pm 9.6 \times 10^{0}$) over the whole sampling period compared to water ($6.2 \times 10^{-3} \pm 1.1 \times 10^{-2}$). The ratio of coliphages to *E. coli* remained unchanged in biofilms and sediments after the rainfall, but decreased by one order of magnitude in water (data not shown).

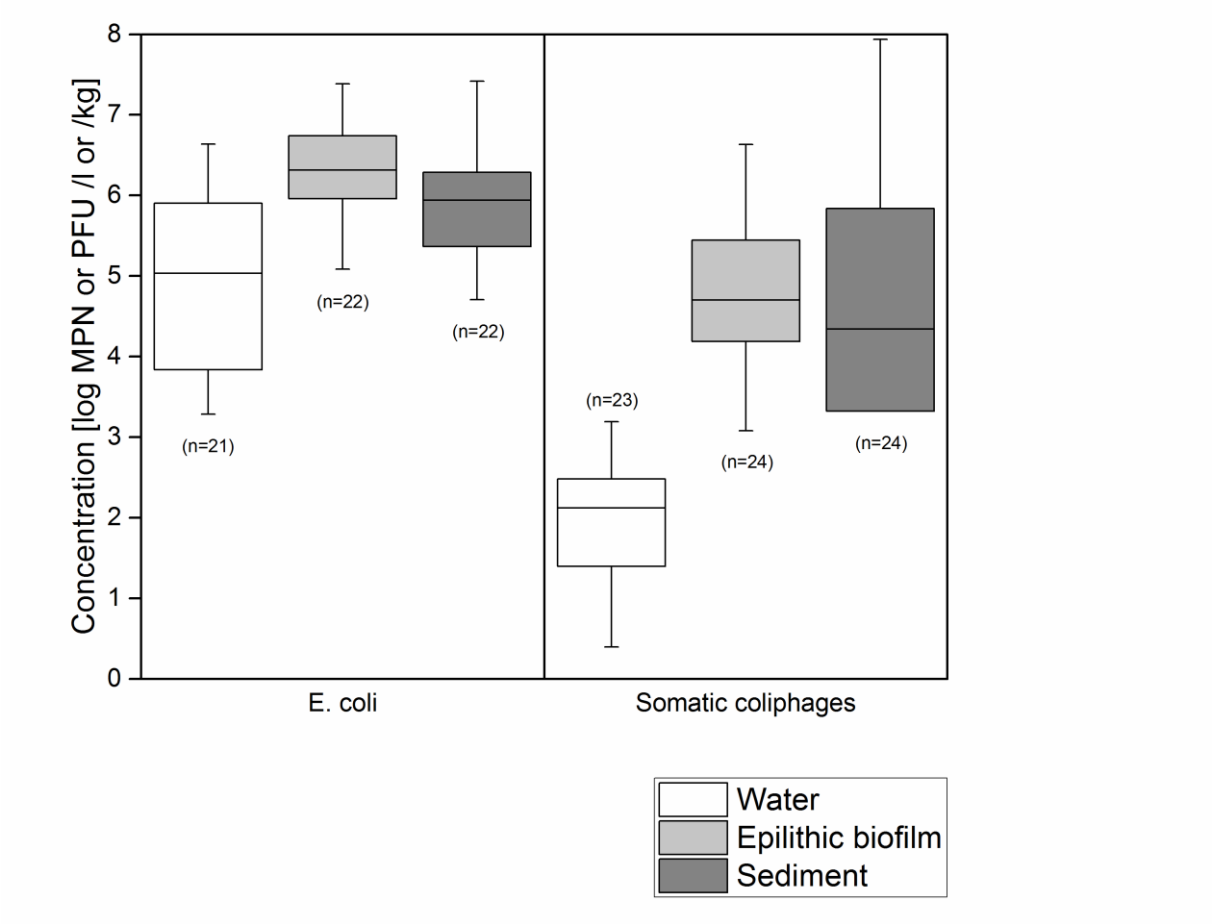

Figure 14: Boxplots for E. coli and somatic coliphages at the three sampling sites on eight sampling dates. Concentrations given as most probable number (MPN), plaque forming units (PFU) per liter of water or kg (wet weight) biofilm or sediment. n = number of samples used for quantification. The boundaries of the boxes indicate the 25th and 75th percentile, the median is shown as a line in the box, dashes indicate minimum and maximum values. (Mackowiak et al., 2018)

5.1.1.4 Occurrence of enteric viruses

In order to determine the distribution of enteric viruses in water, epilithic biofilms and sediments of the River Ruhr, HAdV, EV, RoV and NoV GII were quantified via qPCR and HAdV, EV and RoV additionally via cell culture-based techniques. Enteric viruses were detected in surface water, epilithic biofilms and sediments, the accumulation effect as seen for *E. coli* and somatic coliphages was not observed. Using qPCR, 21 from 24 samples were positive for HAdV, including two samples with a concentration below the limit of quantification, but above the limit of detection. The mean concentration of HAdV was $2.9 \times 10^3 \pm 3.4 \times 10^3$ gen.eq./l. From these samples, 14 samples and one qPCR-negative were positive in cell culture assays with a mean concentration of $6.5 \times 10^2 \pm 1.3 \times 10^3$ $TCID_{50}$ per liter. Five samples were positive for EV with a mean concentration of 9.3×10^3

$\pm$ 1.2 x 10^4 gen.eq./l, using qPCR. Four of these samples and one qPCR-negative sample were positive in cell culture (mean $TCID_{50}$ = 2.4 x 10^1 $\pm$ 7.3 x 10^0 l^{-1}). In 4 out of 24 samples, RoV could be detected using qPCR (mean concentration = 1.4 x 10^4 $\pm$ 1.4 x 10^4 gen.eq./l), two of the positive samples and one qPCR-negative sample were positive using cell culture methods (mean $TCID_{50}$ = 2.1 x 10^1 $\pm$ 8.0 x 10^0 l^{-1}). NoV GII was only detected in two water samples (mean concentration = 1.9 x 10^4 $\pm$ 2.5 x 10^4 gen.eq./l) by qPCR. Overall, the prevalence of enteric viruses in water was HAdV>EV>RoV>NoV GII. Using either qPCR or cell culture, five samples were positive for HAdV, EV and RoV at the same time.

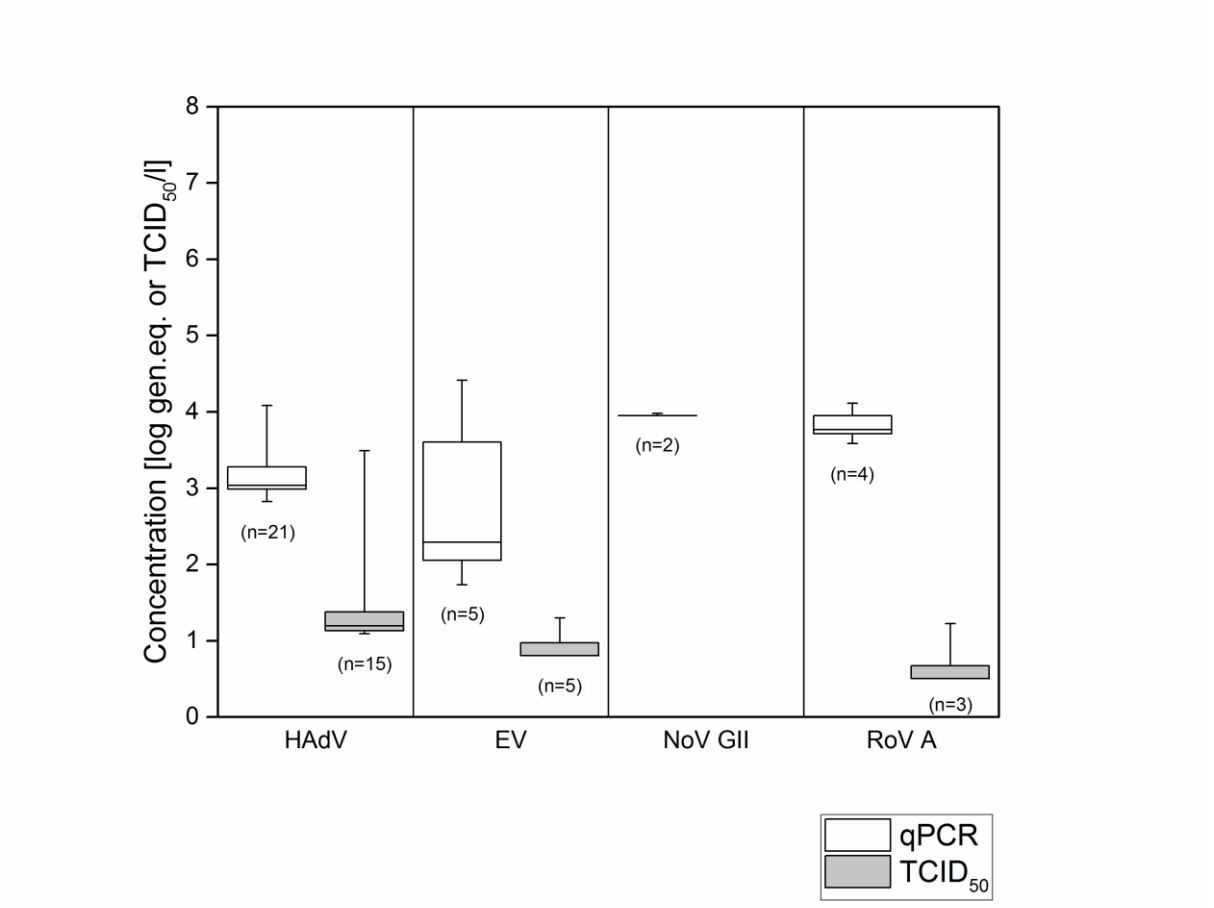

Figure 15: Boxplots for enteric viruses in water at the three sampling sites on eight sampling dates. Concentrations given as genome equivalents (gen.eq.) or $TCID_{50}$ per liter of water. n=number of samples above the limit of quantification. The boundaries of the boxes indicate the 25th and 75th percentile, the median is shown as a line in the box, dashes indicate minimum and maximum values. (Mackowiak et al., 2018)

In case of epilithic biofilms and sediments, no quantitative data were obtained. For all viruses tested, concentrations were below the limit of quantification using qPCR (7.5 x 10^4 gen.eq./kg sediment or 1.5 x 10^5 gen.eq./kg epilithic biofilm, respectively), it was only possible to determine presence or absence of viral DNA or RNA. In case of HAdV, 13 out of 24 samples

from epilithic biofilm were positive, and 11 samples were below the limit of detection (3.0×10^4 gen.eq./kg). For sediments, 12 of 24 samples were positive, the other half of the samples was below the LOD (1.5×10^4 gen.eq./kg). One sample from each, epilithic biofilm and sediment, was tested positive for EV RNA, both were also positive for HAdV. The residual 23 samples were negative for enterovirus and all 24 samples were negative for NoV GII and RoV. We did not detect enteric viruses using cell culture methods. Thus, the prevalence of enteric viruses in biofilms and sediments based on qPCR results was HAdV>EV>RoV, NoV GII. The concentration data of all microbial parameters determined in this study are summarized in boxplot diagrams (Figure 14, Figure 15). No trend was found for any of the observed parameters concerning the sampling point (data not shown). Thus, all data were pooled for analysis.

5.1.1.5 Correlation among environmental and microbial parameters

To estimate the potential influence of the enhanced rainfall which occurred during the period of sampling, two groups of samples (before and after the period of enhanced rainfall) were defined and compared the parameters determined in this study. Two-sample t-Tests were performed to assess whether differences were significant assuming that a *p*-value below 0.05 indicates a significant difference between the two datasets. The mean flow rate increased significantly after the enhanced rainfall, whereas the mean water temperature decreased. Moreover, the concentrations of *E. coli* and coliphages in water increased significantly after the rainfall, but concentrations in biofilms and sediments were not significantly different. Besides, the ratio of coliphages to *E. coli* in water samples decreased after the rainfall indicating that the increase of the *E. coli* concentration in water was higher than for coliphages. In order to test potential correlations between the parameters measured in this study, Pearson product-moment correlation coefficients (PPMCC) were determined for both coliphages and enteric viruses with quantitative data (Table 10). In case of *E. coli*, one water sample was above the range of quantification, thus this sample was excluded from analysis. Correlations for parameters with less than 50 % of the samples above the limit of quantification (LOQ) were excluded from this calculation. Correlations with a *p*-value below 0.05 were assumed significant between the parameters. The water temperature showed a negative correlation with the flow rate (PPMCC = -0.57). The concentrations of *E. coli*, somatic coliphages and HAdV in water were strongly dependent on the flow rate, whereas the concentrations in epilithic biofilms and sediments were independent from the flow rate. The

correlation between the concentration of coliphages in water and the flow rate showed the highest PPMCC of 0.95. Moreover, the concentrations of *E. coli*, HAdV and somatic coliphages in water had positive linear relationships between each other. Interestingly, a significant correlation between the concentration of *E. coli* in epilithic biofilms and HAdV in water was observed (PPMCC = 0.53). Also the concentrations of coliphages in epilithic biofilms and *E. coli* in sediments were correlated (PPMCC = 0.53).

Table 10: Pearson product-moment correlation coefficients (PPMCCs) for all parameters with quantitative data in this study. Significant correlations ($p < 0.05$) are highlighted in bold. Analysis is based on pooled data from all sampling sites. (Mackowiak et al., 2018)

	Flow rate	Water temperature	*E. coli* water	*E. coli* Biofilm	*E. coli* sediment	Coliphages water	Coliphages biofilm	Coliphages sediment	HAdV water (qPCR)	HAdV water ($TCID_{50}$)
Flow rate	-	**-0.57**	**0.69**	0.39	-0.11	**0.95**	0.06	0.05	**0.54**	0.32
Water temperature	**-0.57**	-	-0.27	-0.14	-0.05	**-0.49**	-0.03	-0.34	-0.34	**-0.43**
E. coli water	**0.69**	-0.27	-	0.40	-0.07	**0.66**	0.31	-0.12	**0.62**	0.32
E. coli biofilm	0.39	-0.14	0.40	-	-0.10	0.30	0.04	0.26	**0.53**	0.24
E. coli sediment	-0.11	-0.05	-0.07	-0.10	-	-0.11	**0.53**	0.02	0.12	0.29
Coliphages water	**0.95**	**-0.49**	**0.66**	0.30	-0.11	-	0.06	-0.07	**0.55**	0.26
Coliphages biofilm	0.06	-0.03	0.31	0.04	**0.53**	0.06	-	-0.02	0.11	-0.16
Coliphages sediment	0.05	-0.34	-0.12	0.26	0.02	-0.07	-0.02	-	-0.10	-0.02
HAdV water (qPCR)	**0.54**	-0.34	**0.62**	**0.53**	0.12	**0.55**	0.11	-0.10	-	**0.58**
HAdV water ($TCID_{50}$)	0.32	**-0.43**	0.32	0.24	0.29	0.26	-0.16	-0.02	**0.58**	-

5.1.2 Detection of viruses in drinking water systems for animals

In this field study, the occurrence of somatic coliphages and animal pathogenic viruses was determined in drinking water systems for animals of different piglet breeding farms in Germany. This study was chosen as example for a technical water system with expected fecal pollution. To determine the distribution of viruses, water and biofilms in the drinking water system were sampled periodically during this study and viruses were quantified using plaque assay for coliphages and qPCR for selected animal pathogenic viruses.

5.1.2.1 Evaluation of the concentration method

In order to quantify somatic coliphages and animal pathogenic viruses in drinking water and drinking water biofilms from drinking water systems for animals, a method to concentrate viruses from the samples had to be found. In this work, a concentration method based on PEG precipitation was used. To evaluate this method, spiking experiments were performed using HAdV, MNV and coliphage φX174, which were available in the laboratory. All three target viruses were spiked to 45 ml of deionized water and subsequently samples were concentrated via PEG concentration. Finally, the viruses were quantified by qPCR, coliphage φX174 was additionally quantified using the plaque assay. Recovery rates were calculated by dividing the respective virus concentration by the virus concentration in a control sample with the same number of viruses spiked into 3 ml of DPBS.

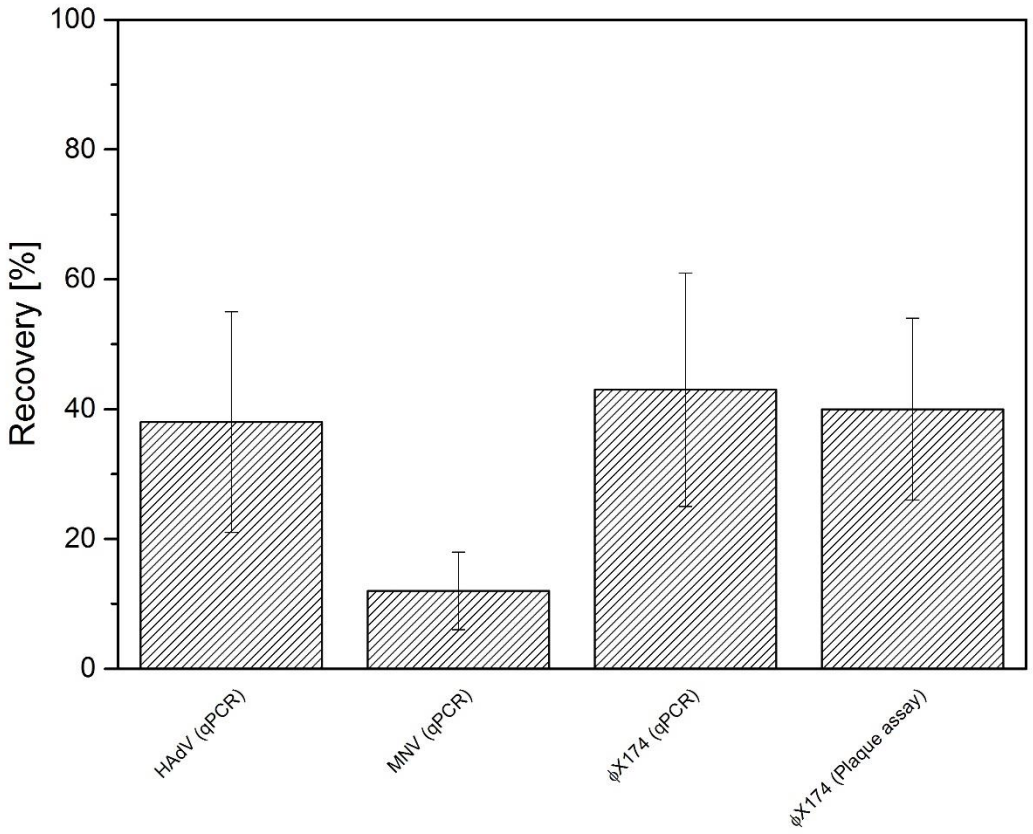

Figure 16: Average recovery rates (n=9) for the concentration step. Recovery was calculated by dividing the concentration in each concentrated sample by the concentration in the respective positive control, which consisted of 3 ml DPBS with the same number of viruses. Error bars indicate the standard deviation.

The average recovery rates were 38 ± 17 % for HAdV, 12 ± 6 % for MNV and 43 ± 18 % for coliphage φX174, using qPCR, respectively (Figure 16). The recovery of infectious coliphages, determined via plaque assay, was comparable to the qPCR result (40 ± 14 %). In conclusion, the recovery rate was similar for somatic coliphage φX174 and HAdV, whereas it was lower for the RNA virus MNV.

5.1.2.2 Occurrence of viruses in water and biofilms from drinking water systems for animals

During this field study, a total number of 84 samples, including 25 water samples (9 from inflowing water and 16 from distribution systems in the piglet breeding farms) and 59 biofilm samples were analyzed for the occurrence of somatic coliphages and animal pathogenic viruses (PAdV, HEV, PRRSV and PCV2). Coliphages were quantified culturally (DIN EN ISO 10705-2), animal pathogenic viruses were analyzed by qPCR. In total, 14/84 samples (16.7 %) were positive for somatic coliphages, including 2/25 (8.0 %) water and 12/59 (20.3 %) biofilm samples, respectively. The average concentration was $4.8 \times 10^1 \pm 6.4 \times 10^1$ PFU/ml in water, and $3.8 \times 10^1 \pm 8.5 \times 10^1$ PFU/cm^2 in biofilm samples. 16/84 samples (19.0 %), including 1/25 (4.0 %) water and 15/59 (25.4 %) biofilm samples, were positive for PAdV, using qPCR. The average concentration in biofilms was $1.9 \times 10^4 \pm 3.5 \times 10^4$ gen.eq./cm^2, the positive water sample showed a concentration of 8.1×10^2 gen.eq./ml. In the inflowing water, somatic coliphages or PAdV were not detected. HEV positive qPCR-results were obtained in 52/84 (61.9 %) of all samples, with 16/25 (64.0 %) water and 36/59 (61.0 %) biofilm samples tested positive with an average concentration of $7.0 \times 10^5 \pm 1.6 \times 10^6$ gen.eq./ml for water samples and $5.6 \times 10^4 \pm 2.8 \times 10^5$ gen.eq./cm^2 for biofilm samples, respectively. In case of HEV, positive water samples included samples from the inflowing water and samples from the distribution system. For inflowing water, 6/9 (66.7 %) samples were positive with an average concentration of $8.3 \times 10^4 + 1.8 \times 10^5$ gen.eq./ml.

The two other viruses (PRRSV and PCV2) were not detected in this study. The results for somatic coliphages, PAdV and HEV from the five different piglet breeding farms are summarized in Table 11.

Table 11: Percentage of positive samples, total number of positive samples, mean concentration of positive samples (± standard deviation) for somatic coliphages (PFU/ml water or PFU/cm²), PAdV and HEV (gen.eq./ml water or gen.eq./cm²) at the five different piglet breeding farms sampled in this study; n_+=number of positive samples; n_{total}=total number of samples; n.d.: not detected. Because somatic coliphages and PAdV were not detected in samples from inflowing waters, this table only includes water samples from the distribution systems.

		Somatic coliphages		PAdV		HEV	
Farm	Sample type	% positive (n_+/n_{total})	c [pfu/ml or PFU/cm²]	% positive (n_+/n_{total})	c [gen.eq./ml or gen.eq./cm²]	% positive (n_+/n_{total})	c [gen.eq./ml or gen.eq./cm²]
1	Water	14 (1/7)	2.7×10^0	0 (0/7)	n.d.	57 (4/7)*	$2.8 \times 10^6 \pm 1.9 \times 10^6$
	Biofilm	13 (1/8)	6.0×10^1	38 (3/8)	$8.1 \times 10^4 \pm 2.0 \times 10^4$	38 (3/8)*	$5.8 \times 10^5 \pm 8.0 \times 10^5$
2	Water	0 (0/2)	n.d.	0 (0/2)	n.d.	50 (1/2)*	8.7×10^3
	Biofilm	19 (3/16)	$7.3 \times 10^0 \pm 1.2 \times 10^1$	0 (0/16)	n.d.	63 (10/16)*	$7.5 \times 10^3 \pm 1.2 \times 10^4$
3	Water	0 (0/1)	n.d.	0 (0/1)	n.d.	100 (1/1)*	1.4×10^2
	Biofilm	13 (1/8)	2.3×10^0	88 (7/8)	$2.4 \times 10^3 \pm 4.4 \times 10^3$	50 (4/8)*	$7.0 \times 10^2 \pm 7.9 \times 10^2$
4	Water	25 (1/4)	9.3×10^1	25 (1/4)	8.1×10^2	0 (0/4)*	n.d.
	Biofilm	46 (6/13)	$6.3 \times 10^1 \pm 1.2 \times 10^2$	23 (3/13)	$2.3 \times 10^2 \pm 2.1 \times 10^2$	54 (7/13)*	$2.6 \times 10^3 \pm 4.5 \times 10^3$
5	Water	0 (0/2)	n.d.	0 (0/2)	n.d.	100 (2/2)*	$1.6 \times 10^4 \pm 1.2 \times 10^4$
	Biofilm	7 (1/14)	1.1×10^0	14 (2/14)	$7.2 \times 10^2 \pm 4.7 \times 10^2$	86 (12/14)*	$5.3 \times 10^4 \pm 1.9 \times 10^4$

*Results obtained for HEV are questionable, because they potentially include false-positive result

Besides water and biofilm samples, negative controls were included for the virus concentration step (45 ml distilled water), DNA/RNA extraction (200 µl DPBS) and qPCR run (PCR water). While negative controls for PAdV did not result in a positive qPCR signal, some negative controls were sporadically positive for HEV. In order to determine, whether false positive results were obtained, PCR products from samples, standards and negative controls were analyzed via agarose gel electrophoresis (Figure 17).

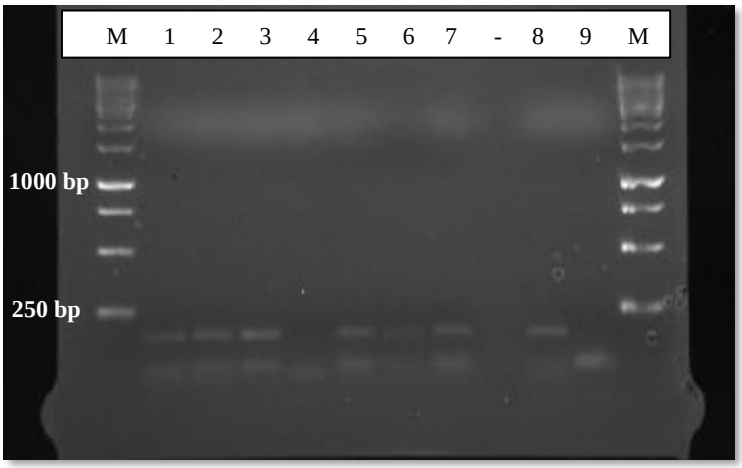

Figure 17: Example of an agarose gel from HEV qPCR products. M=marker, 1,2,8: negative controls, 3-7: positive samples, 9: positive control.

The analysis of the HEV PCR products via agarose gel electrophoresis revealed that more than one PCR product was formed. For samples and some negative controls, one or two bands below fragment size 250 bp were observed, whereas the positive control only displayed one band (Figure 17).

5.2 Laboratory experiments

5.2.1 Interaction of enteric viruses and somatic coliphages with drinking water biofilms

In the following part of this work, the retention of selected model viruses (HAdV, MNV and coliphage φX174) in drinking water biofilms was studied under different physicochemical and hydraulic conditions. Drinking water biofilms were grown for 14 d on EPDM in stainless steel reactors supplied with drinking water. During biofilm growth, the drinking water was analyzed to determine variations in temperature, pH and concentrations of calcium and magnesium. Subsequently, viruses were added simultaneously to the water phase and reactors were further incubated for 7 d under either stagnant or flow conditions. In case of stagnant conditions, virus retention was assessed using drinking water with and without addition of calcium to determine the effect of water hardness on virus distribution. After 1 and 7 d of incubation with viruses, water and biofilms were sampled and viruses were quantified using qPCR, cell culture and plaque assay. Moreover, biofilms were characterized by microbiological methods and the determination of the wet weight.

5.2.1.1 Drinking water characteristics

During growth of drinking water biofilms, physicochemical parameters were measured twice per day to assess the variations in temperature, pH, calcium and magnesium concentrations in the drinking water (Table 12). The water temperature ranged from 12.1 °C to 21.9 °C (with n = 92 samples and 12 individual reactor runs) throughout this study. While variation between different experimental runs was relatively high, depending on season of the year, the variation within each reactor run was within 3.2 °C (data not shown). It should be noted that the water temperature remained constant at 20 °C (room temperature) after the addition of viruses, because the system was not supplied with water from the distribution system during incubation. Instead, reactors were incubated under either stagnant conditions or run in circular flow. The average pH of the inflowing drinking water was 7.8 $\pm$ 0.3. According to the local drinking water supplier, drinking water is only chlorinated on demand (Stadtwerke Essen AG, 2018), free chlorine was not measured in any of the water samples (data not shown) during this study. One parameter that can vary significantly between drinking water of different sources is the water hardness. It is known that calcium can influence the structure and mechanical stability of aquatic biofilms (Kelly et al., 2014; Korstgens et al., 2001; Shen et al., 2018), moreover divalent cations could potentially affect the adsorption of viruses. Therefore,

concentrations of the divalent cations Ca^{2+} and Mg^{2+} were assessed via ion chromatography. The average concentrations in drinking water were 38.1 ± 3.8 mg/l and 6.7 ± 1.0 mg/l for Ca^{2+} and Mg^{2+}, respectively, which implies that the water can be regarded as "soft" according to the German regulation (BMJV, 2007). For some reactor runs, the drinking water was supplied with calcium carbonate to create artificially hardened drinking water. For this hardened water, the average Ca^{2+} concentration was 120.8 ± 5.8 mg/l, which classifies this water as "hard" according to German regulation (BMJV, 2007).

Table 12: Physicochemical parameters of the inflowing drinking water used for drinking water biofilm growth in this study.

Parameter	n	Mean	SD	Min	Max
T [°C]	92	17.8	3.0	12.1	21.9
pH	92	7.8	0.3	7.0	8.3
Ca^{2+} [mg/l]	77	38.1	3.8	30.5	45.4
Mg^{2+} [mg/l]	77	6.7	1.0	4.6	8.5

5.2.1.2 Growth of drinking water biofilms

In this work, drinking water biofilms were grown on EPDM coupons under flow conditions for 14 d. Subsequently, HAdV, MNV and coliphage φX174 were added and biofilms were further incubated under either stagnant or flow conditions with for 1 d or 7 d. The biomass per square centimeter was determined at the end of the respective incubation period (i.e. after 15 or 21 d total growth) by weighing each coupon and subtracting the weight of the coupon before biofilm growth. The average biomass throughout all samples was 11.8 ± 2.2 mg/cm^2 after 15 d and 18.3 ± 12.9 mg/cm^2 after 21 d of growth, ranging from 5.8 mg/cm^2 to 67.9 mg/cm^2. There was a clear difference in biofilm mass for coupons removed after 21 d depending on whether they were incubated under stagnant (9.9 ± 1.9 mg/cm^2) or flow (26.7 ± 13.9 mg/cm^2) conditions after the addition of viruses. In addition, it was also visible macroscopically that biofilms had an increased biomass after the incubation under circular flow compared to stagnant conditions. It is worth to note that the water turbidity increased during circular flow. No significant difference was observed in biofilm biomass between samples from experiments with and without the addition of calcium to the drinking water (data not shown). Total cell counts and heterotrophic plate counts of biofilms after 15 d and

21 d of incubation were analyzed for the two incubation conditions (4 individual reactors under stagnant conditions, 2 individual reactors under flow conditions). The average total cell count was $2.3 \times 10^8 \pm 6.6 \times 10^7$ cells/cm^2, with no significant differences between biofilms after 15 d or 21 d of growth (data not shown). The total cell count after 21 d was higher after incubation under stagnant conditions ($2.7 \times 10^8 \pm 1.8 \times 10^8$ cells/cm^2) compared to flow conditions ($6.1 \times 10^7 \pm 3.0 \times 10^7$ cells/cm^2), whereas the difference was not observed after 15 d of growth (i.e. 1 d incubation under the respective condition). The average heterotrophic plate count was $1.6 \times 10^7 \pm 3.4 \times 10^6$ CFU/cm^2 after 15 d of growth and decreased to $6.7 \times 10^6 \pm 5.6 \times 10^6$ CFU/cm^2 after 21 d. No significant differences were observed when comparing stagnant or flow conditions (data not shown). Based on total cell count and heterotrophic plate count, the percentage of culturable bacteria was calculated. For biofilms incubated under stagnant conditions, the percentage decreased from 6.4 ± 1.8 % after 15 d to 1.8 ± 0.9 % after 21 d of growth, whereas it increased from 9.2 ± 0.0 % to 14.1 ± 0.3 % under flow conditions within the same period.

Biofilms were not only formed on EPDM coupons, but also inside of tubing used during biofilm growth, which was observed macroscopically. To quantify the extent of (unwanted) biofilm formation on tubing, all tubes were weighed before and after growth. While the surface area of the EPDM coupons was 40 % of the overall surface area allowing biofilm growth (EPDM coupons and tubing), the biofilm that developed on EPDM throughout the experiments contributed to 50.0 ± 14.3 % of the overall wet weight of biofilms (data not shown).

5.2.1.3 Fate of viruses in drinking water

After the addition of viruses to the water, reactors were incubated for 7 d under the respective conditions. During incubation, the concentrations of the three target viruses (coliphage φX174, HAdV and MNV) were assessed using both PCR-based methods and plaque assay or cell culture. Water samples were taken at the beginning of the incubation period, after 1 d and 7 d of incubation. The first sample (t=0) was taken 30 minutes after the addition of viruses to allow sufficient mixing in the reactors. The absolute concentrations of viruses at the beginning of each experiment differed significantly between individual reactor runs, ranging from 2.87×10^5 to 3.69×10^8 gen.eq./ml for HAdV, from 5.13×10^3 to 4.15×10^6 gen.eq./ml for MNV and from 8.83×10^4 to 9.49×10^6 gen.eq./ml for coliphage φX174, respectively. The

reason for these differences was that first experiments with low initial virus concentrations revealed results below the limit of detection for some samples. Thus, starting concentrations were increased for later experiments. For comparison, the concentration of viruses in water at each time point was compared to the concentration at the beginning of the experiment and the logarithmic reduction was determined (Figure 18).

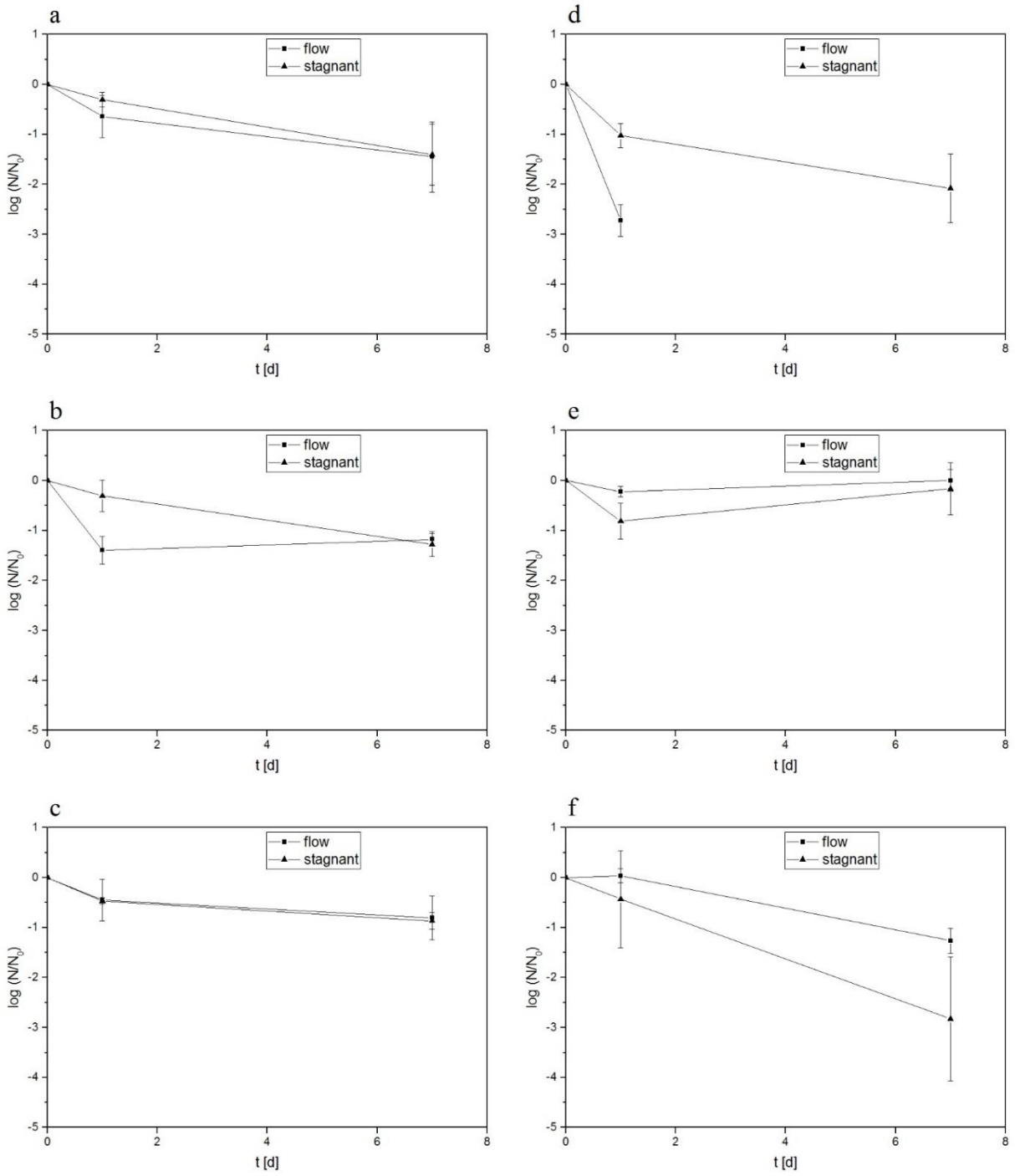

Figure 18: Average logarithmic ratio of the virus concentration at each time point (N) and the virus concentration at the beginning of the incubation period (N_0) for HAdV, MNV and coliphage φX174 in water during incubation in stainless steel reactors under stagnant (black triangles) and flow conditions (black squares) for 7 d at room temperature. ***a****: HAdV, analysis via qPCR (stagnant: n=6, flow: n=6);* ***b****: MNV, analysis via qPCR (stagnant: n=5, flow: n=2);* ***c****: Coliphage φX174, analysis via qPCR (stagnant: n=6, flow: n=6);* ***d****: HAdV, analysis via cell culture (stagnant: n=6, flow: n=2);* ***e****: MNV, analysis via cell culture (stagnant: n=6, flow: n=2);* ***f****: Coliphage φX174, analysis via plaque assay (stagnant: n=4, flow: n=2). Error bars indicate the standard deviation of each dataset.*

For HAdV, the concentration determined by qPCR decreased by 1.4 ± 0.6 log units within the 7 d incubation period, with no significant differences between stagnant and flow conditions. The reduction for infectious HAdV in water after 7 d was 2.1 ± 0.7 log units, with clear differences depending on the flow conditions during incubation. Under flow conditions, the concentration of infectious HAdV, determined culturally, decreased by 2.7 ± 0.3 log units within one day of incubation, whereas it decreased by 1.0 ± 0.2 log unit under stagnant conditions. After 7 d, the concentration of infectious HAdV was below the limit of detection for samples taken under flow conditions, whereas the concentration decreased by 2.1 ± 0.7 log units under stagnant conditions.

For MNV, the concentration determined by qPCR decreased by 1.1 ± 0.3 log units within 7 d under stagnant conditions. In contrast to that, it decreased rapidly by 1.4 ± 0.3 after 1 d under flow conditions and remained constant until day 7 of the incubation period. Concerning infectious MNV, the concentration decreased by less than 1 log unit after 1 d for both flow conditions, but no decrease was observed after 7 d.

In case of somatic coliphage φX174, the virus concentration in water determined by qPCR decreased by 0.8 ± 0.3 log unit within one week of incubation. No significant differences were observed for concentrations comparing the two flow conditions. The number of infectious coliphages, determined by plaque assay, decreased for both conditions, the decrease after 7 d was higher for stagnant conditions (2.8 ± 1.2 log units) than for flow conditions (1.3 ± 0.3 log units). It should be noted that the standard deviation was comparatively high for stagnant conditions though.

The average reduction rates were calculated for all viruses and flow conditions based on the logarithmic reductions 1 d and 7 d after the addition of viruses (Table 13).

Table 13: Average reduction rates r calculated for the different viruses in water. Rates were determined based on all data from experiments under stagnant and flow conditions according to equation 1; n: number of experiments used for calculation; SD: standard deviation.

		qPCR			**cell culture / plaque assay**	
Virus	n	r [log/d] (mean)	r [log/d] (SD)	n	r [log/d] (mean)	r [log/d] (SD)
HAdV	12	-0.1963	0.0873	6	-0.1757*	0.1304*
MNV	9	-0.1579	0.1560	8	0.0911	0.0726
φX174	12	-0.1336	0.1316	6	-0.3382	0.1232

** No reduction rate could be calculated for flow conditions.*

In conclusion, reduction rates show that the decrease of genome concentration in water was highest for HAdV, followed by MNV and coliphage φX174. For viral infectivity, the reduction rate was highest for coliphage φX174, followed by HAdV. In case of MNV, no reduction in concentrations of infectious viruses was observed, which caused the reduction to be positive. It is important to mention that experiments for infectious HAdV incubated under flow conditions were excluded from calculations because the concentration of infectious HAdV was below the limit of detection after 7 d of incubation. Thus, the real reduction rate would be much higher for HAdV.

5.2.1.4 Incorporation of viruses into drinking water biofilms

5.2.1.4.1 Quantification of viruses by qPCR

Besides virus concentrations in water, also concentrations in the drinking water biofilms were assessed after 1 d and 7 d of incubation in order to determine the distribution of the target viruses (HAdV, MNV and coliphage φX174) between water and biofilms. Using qPCR, all three viruses were detected in the biofilm samples of the different incubation conditions. None of the viruses was detected in water or biofilms without prior addition to the system (data not shown), indicating that the inflowing drinking water did not contribute to the concentration of viruses. To evaluate a potential accumulation of viruses in the biofilm, calculations were made assuming that one gram (wet weight) of biofilm corresponds to one milliliter of water. This assumption was done because the EPS matrix of biofilms mainly consists of water (Flemming et al., 2016). The decadic logarithm of the ratio between the virus concentration per gram (wet weight) of biofilm and the concentration per milliliter of water was used as indicator for virus distribution between the two phases (Figure 19). Consequently, a $\log\left(\frac{c_{biofilm}}{c_{water}}\right) > 0$ should be estimated for an accumulation in the biofilm, a $\log\left(\frac{c_{biofilm}}{c_{water}}\right) < 0$ for an accumulation in the water phase, whereas a $\log\left(\frac{c_{biofilm}}{c_{water}}\right)$ of 0 would result from an even distribution between water and biofilm. As for water samples, biofilm samples were collected 1 d and 7 d after the addition of viruses.

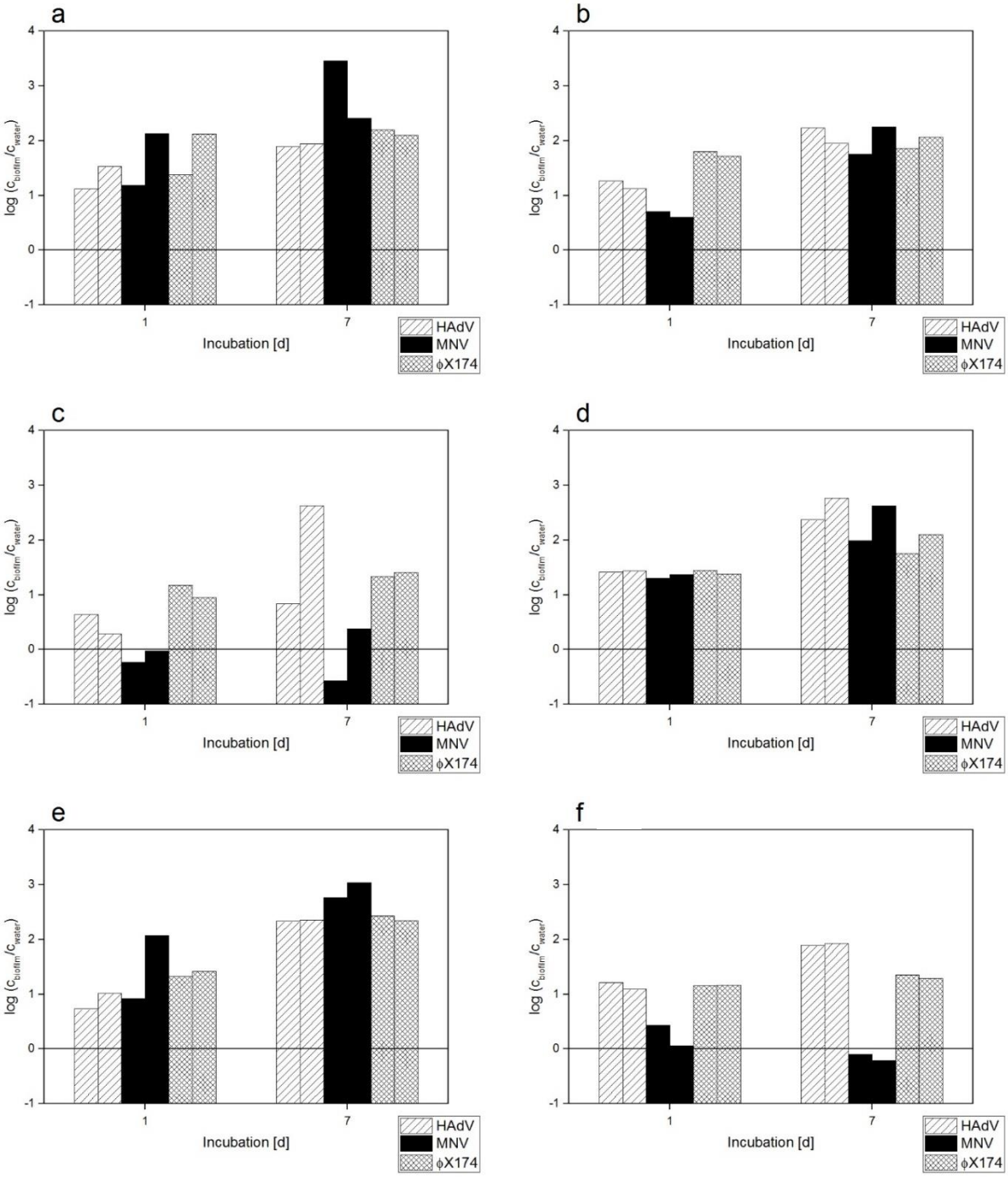

Figure 19: Logarithmic ratio of the virus concentration (determined by qPCR) in water and biofilm (log ($c_{biofilm}/c_{water}$)) for HAdV, MNV and coliphage φX174 after 1d and 7d of incubation at the respective experimental condition. Each graph shows results from a single experiment with two individual reactors in parallel, represented by two bars for each virus. ***a-c****: stagnant conditions, soft water;* ***d, e****: stagnant conditions, hard water;* ***f****: flow conditions, soft water.*

In most of the experiments, the $\log\left(\frac{c_{biofilm}}{c_{water}}\right)$ was between 1 and 3 for all three viruses after 1 d and 7 d of incubation showing that the virus concentration determined by qPCR was higher in the biofilm than in the surrounding water. In two experiments, no such trend was observed for MNV (Figure 19c, f). For almost all datasets, the $\log\left(\frac{c_{biofilm}}{c_{water}}\right)$ increased from 1 d to 7 d of incubation, thus no steady state was reached after 24 h of incubation. The highest accumulation of viruses was found for MNV after 7 d of incubation under stagnant conditions in soft water (Figure 19a), the concentration in the biofilm was approximately 3.5 log units higher than in the water. Comparing the different experimental conditions, the incubation under flow conditions resulted in the least accumulation of the target viruses (Figure 19f), although one experiment under stagnant conditions showed comparable results (Figure 19c). No clear differences were observed between soft and hard water or the different types of viruses, except the two cases without accumulation of MNV, as mentioned above. It turned out that the variation of the $\log\left(\frac{c_{biofilm}}{c_{water}}\right)$ for individual viruses was relatively low between biological duplicates of the same experiment, whereas the variation was sometimes several log units between different experiments – even when the experimental conditions were the same. In conclusion, viral DNA or RNA of all three viruses accumulated in drinking water biofilms by up to 3.5 log units compared to the water phase, regardless of virus type, flow conditions or water hardness. Moreover, high variation between different experiments impedes predictions about the exact distribution between water and biofilms.

Besides biofilms on EPDM coupons, biofilms also grew inside of tubing material at the influent and effluent of the reactors. On a random basis, biofilms grown in tubing was also analyzed for the occurrence of the target viruses from this work showing that viruses accumulated to a comparable extent into these biofilms (data not shown).

5.2.1.4.2 Quantification of infectious viruses

The detection of viruses by qPCR does not provide information about the concentration of infectious viruses. Therefore, the biofilm samples were not only analyzed by qPCR, but also culture-based methods to determine the number of infectious viruses. HAdV and MNV were quantified by the $TCID_{50}$ assay using eukaryotic cell lines. Infectious coliphages φX174 were quantified using the plaque assay.

Confirming qPCR results, also infectious viruses accumulated in biofilms under most of the experimental conditions, but high variance between different experiments was observed. Interestingly, several experiments did not reveal an accumulation in the biofilm. In many cases, it was not possible to calculate the $\log\left(\frac{c_{biofilm}}{c_{water}}\right)$ values, because the concentration of infectious viruses in either water or biofilm was below the limit of quantification. As already found with qPCR, infectious viruses showed the weakest tendency to associate with biofilms under flow conditions. For infectious HAdV, no clear trend was found for accumulation in soft water, whereas a 2-3.5 log unit higher concentrations were observed in biofilms after 7 d of incubation in hard water (Figure 20d, e). Also for coliphage φX174, the highest accumulation was found in hard water, although this trend was not as clear as for HAdV. For MNV, accumulation by 1-3 log units was observed for all experimental conditions, however it should be noted that the cell line used for MNV quantification (RAW 264.7) also displayed cytopathic effect for water and biofilm samples without the addition of viruses. This fact implies that results for MNV should be regarded with caution because the accumulation effect might be due to false positive results in the $TCID_{50}$ assay.

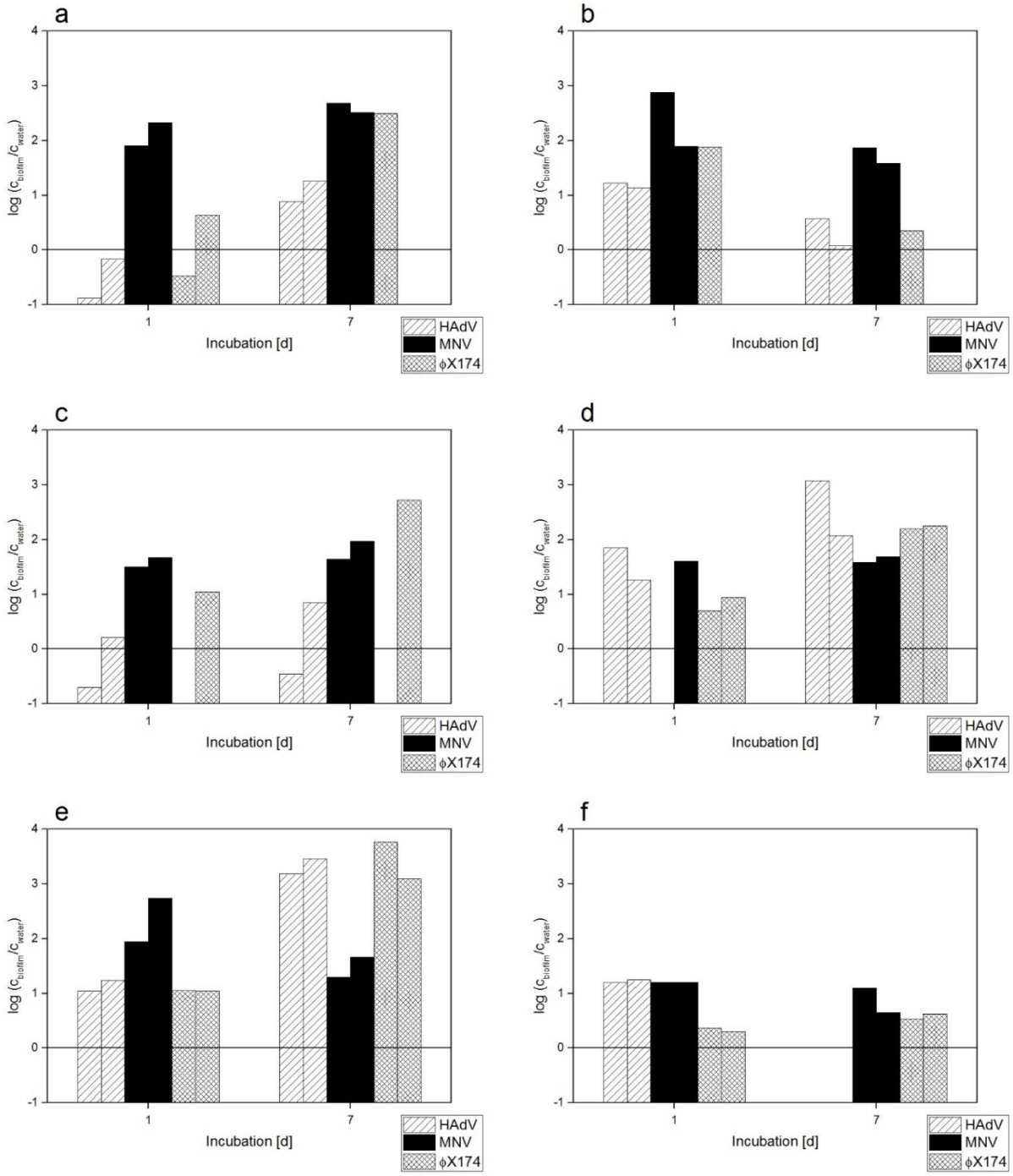

Figure 20: Logarithmic ratio of the virus concentration (determined by cell culture and plaque assay) in water and biofilm (log ($c_{biofilm}/c_{water}$)) for HAdV, MNV and coliphage φX174 after 1d and 7d of incubation at the respective experimental condition. Each graph shows results from a single experiment with two individual reactors in parallel, represented by two bars for each virus. ***a-c****: stagnant conditions, soft water;* ***d, e****: stagnant conditions, hard water;* ***f****: flow conditions, soft water.*

5.2.2 Biological interactions of viruses in biofilms

5.2.2.1 Co-cultivation of *A. castellanii* and HAdV

Amoebae are ubiquitously found in aquatic environments and studies showed that they can harbor enteric viruses, potentially providing protection from environmental stressors. In the following part of this work, the role of *A. castellanii* during virus retention in aquatic biofilms should be further elucidated. *A. castellanii* was chosen in this work because a previous study has shown that it can incorporate HAdV (Scheid and Schwarzenberger, 2012). The first aim of this work was to generally verify the uptake of HAdV by *A. castellanii* under co-cultivation. Therefore, amoebae were incubated in cell culture flasks with growth medium containing ~ 10^9 gen.eq./ml HAdV for 7 d at 25 °C. The concentration of HAdV in the growth medium at the beginning of the incubation and after 7 d was determined by qPCR. Additionally, amoebae adhering to the bottom of the flask were analyzed for the presence of HAdV after 7 d of incubation. Within one week of incubation, the concentration of HAdV decreased by 0.4 log units from 7.4 x 10^8 gen.eq./ml to 2.9 x 10^8 $\pm$ 8.5 x 10^7 gen.eq./ml in the medium. Besides medium, also adherent amoebae were tested positive for HAdV with 5.2 x 10^6 $\pm$ 2.6 x 10^5 gen.eq. associated with adherent amoebae. It should be noted that adherent amoebae represent only a small fraction of the overall volume in the culture, thus concentrations between amoebae and medium cannot be compared. Nevertheless, this work has confirmed that *A. castellanii* had taken up HAdV in co-culture. In a next step, attempts were made to show this incorporation with fluorescence microscopy using antibody-labelled HAdV, in order to use this detection method for the analysis of amoebae in drinking water biofilms. Unfortunately, it was not possible to visualize labelled HAdV inside of amoebae in this work (data not shown).

5.2.2.2 Interaction between somatic coliphages and *E. coli* in monospecies biofilms

Somatic coliphages are controversially discussed as indicators for enteric viruses in the aquatic environment. In this context, one major prerequisite is that coliphages cannot multiply in the aquatic environment, despite the presence of their bacterial host *E. coli*. While growth of somatic coliphages in water is assumed to be negligible, no data exist for aquatic biofilms, which could potentially increase the probability of virus infection due to elevated bacterial densities. In order to determine whether the somatic coliphage φX174 could propagate in monospecies biofilms of *E. coli* under environmental conditions, laboratory experiments in a 96-well microtiterplate format were performed. *E. coli* DSM 13127 biofilms were grown for

48 h in LB medium, then the medium with planktonic cells were removed and 10^3 or 10^6 pfu/ml coliphage φX174 was added in LB medium or sterile filtered surface water from the River Ruhr in Essen, Germany. LB medium or water without coliphages served as negative controls. Plates were incubated at 20 °C (to mimic surface water conditions in a temperate climate zone) and 36 °C (as optimal growth temperature for *E. coli*). To quantify the biofilm at the beginning of the incubation, eight wells of each plate were stained directly before the addition of phages. The phage titer in the medium was determined at time points 0, 1 h and 4 h after the addition of phages via plaque assay. At the end of the 4 h incubation period, biofilms were quantified photometrically (Figure 21).

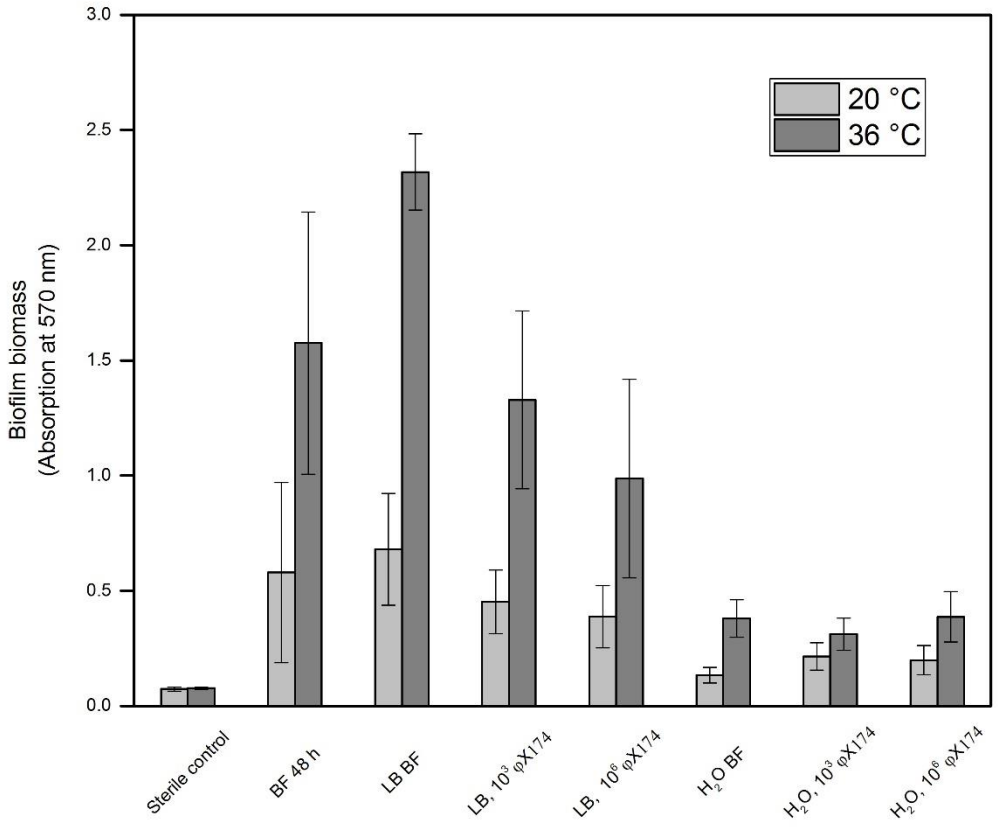

Figure 21: Average absorption (n=8) of dissolved crystal violet at 570 nm. E. coli biofilms were grown at 36 °C for 48 h in LB medium. Subsequently, the planktonic phase was removed and biofilms were incubated with LB medium (LB) or water (H_2O) with 10^3 or 10^6 pfu/ml coliphage φX174 at 20 °C or 36 °C for 4 h. LB medium or water without phages (LB BF and H_2O BF) served as control. After incubation, the planktonic phase was removed and remaining biofilms were stained with crystal violet, which was dissolved with 30 % acetic acid prior analysis. BF 48 h: Biofilm biomass after the bars indicate the standard deviation from eight wells.

Under any condition, the absorption crystal violet at 570 nm, which is proportional to the biofilm biomass, was higher at 36 °C than at 20 °C. The average biofilm biomass for biofilms grown in LB without coliphages at 36 °C increased within the 4 h incubation period (Figure 21). Biofilms exposed to 10^3 pfu/ml coliphage φX174 at 36 °C did not show this increase, biofilms exposed to 10^6 pfu/ml at 36 °C even had less biomass compared to the biofilm before addition of phages (BF 48 h). At 20 °C, these general trends were also observed, however they were not as clear as for biofilms grown at 36 °C. For biofilms grown in sterile filtered surface water, no trend between different phage exposures was observed. It should be noted that all biofilms (including the negative control without phages) exposed to surface water showed an absorption below 0.5, indicating that the biofilm biomass was very low. This detachment of biofilms after 4 h of incubation with surface water was also observed macroscopically.

Comparing the titer of coliphage φX174 for the different conditions, it turned out that only incubation at 36 °C in LB medium showed an increase in coliphage concentration within 4 h. The average titer in LB with a starting concentration of 10^3 pfu/ml increased to 3.5×10^8 PFU/ml, it reached 3.0×10^9 PFU/ml in LB with a starting concentration of 10^6 PFU/ml after 4 h, respectively. For biofilms grown at 20 °C in LB medium or 20 °C and 36 °C in surface water, no propagation of coliphage φX174 was observed after 4 h.

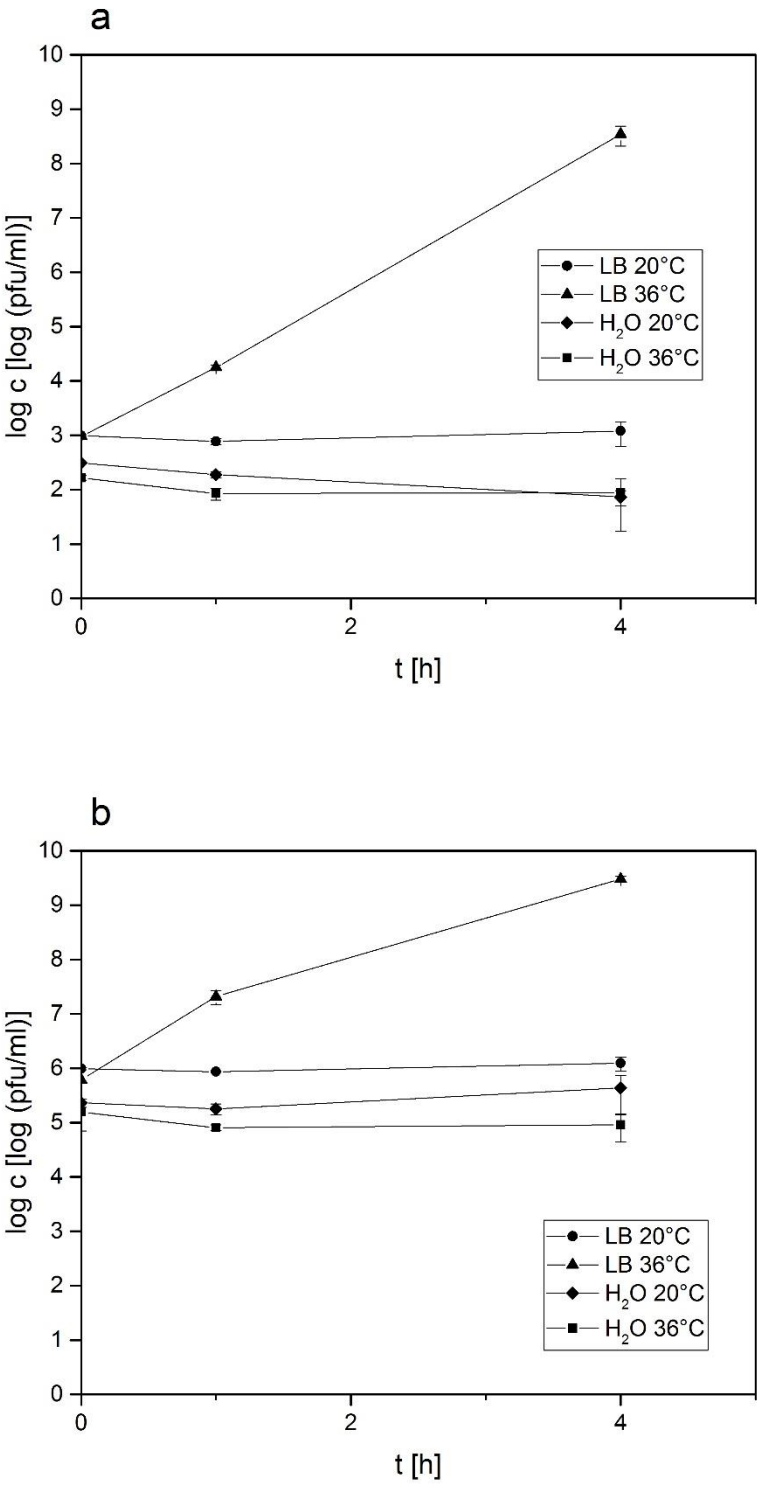

Figure 22: Average concentration (n=3) of infectious coliphage φX174 (as PFU/ml) for different incubation conditions. Error bars indicate the standard deviation of each triplicate. ***a****: Initial concentration of* 10^3 *PFU/ml;* ***b****: Initial concentration of* 10^6 *PFU/ml*

6. Discussion

In this work, the interaction between viruses and aquatic biofilms in the urban water cycle was studied in two field studies and in laboratory scale experiments. First, two field studies were conducted to assess the distribution of viruses in aquatic biofilms of the urban water cycle. In laboratory experiments, the incorporation of viruses was elucidated with artificially grown drinking water biofilms. Moreover, potential biological interactions within the biofilm were studied.

6.1 Field studies

Although laboratory experiments are suitable to study the interaction between viruses and aquatic biofilms under distinct conditions, they do not necessarily reflect realistic natural or technical systems in the urban water cycle. Therefore, two field studies were conducted in order to elucidate the relevance of aquatic biofilms as reservoirs for viruses in the urban water cycle. While one field study focused on epilithic biofilms and sediments of a river as an example for a natural system, the second field study was done to assess the distribution of viruses in water and biofilms from drinking water systems of different piglet breeding farms as an example for technical water systems.

6.1.1 Detection of viruses in water, sediments and epilithic biofilms of the river Ruhr in Essen, Germany

In this field study, the distribution of selected human enteric viruses (HAdV, EV, RoV, NoV GII), somatic coliphages and *E. coli* as bacterial indicator were assessed in water, epilithic biofilms and sediments of an urban river in a densely populated area in Germany. The river Ruhr in Essen was chosen because it is anthropogenically polluted by effluents from wastewater treatment plants, it serves as raw water for drinking water production and is used for recreational activities. A previous study at the River Ruhr demonstrated that enhanced rainfall can cause significant increases in fecal pollution of the water, potentially due to elevated discharges from wastewater treatment plants, combined sewer overflows or increased runoff from agricultural areas (Strathmann et al., 2016). In order to assess the influence of enhanced rainfall on the hygienic situation of water, biofilms and sediments of the river, two different weather conditions were included in this study. A phase with relatively low

precipitation was followed by a period of enhanced rainfall with a significant increase in river flow rate, which allowed the comparison of the microbiological situation in the water column and in biofilms before and after the strong increase in precipitation.

6.1.1.1 Evaluation of virus elution efficiency

In order to determine the occurrence of viruses in epilithic biofilms and sediments from the River Ruhr, a method for virus elution had to be found to isolate viruses from biofilms and sediments. Five different isolation protocols including three different elution buffers and two concentration protocols were tested after spiking ~10^7 gen.eq. HAdV and coliphage φX174 to 10 g (wet weight) of River Ruhr sediment. The tested methods involved various combinations of elution buffers and physicochemical treatments for the separation of viruses from the sample matrix.

From the five different protocols, the protocol based on sonication of samples in elution buffer (1 % (w/v) beef extract, 0.375 % (w/v) glycine, pH 9.4) followed by centrifugation and subsequent PEG concentration of the supernatant was most efficient for the isolation of the two target viruses. The mean recovery rates of this method were 77 % for HAdV and 56 % for coliphage φX174, respectively. These recovery rates are comparable to those obtained by Helmi et al. (2010), who performed recovery experiments for the isolation of coliphage MS2 and coliphage φX174 from 7d-old drinking water biofilms grown on PVC, cement and cast iron. Depending on the material used for biofilm growth, recoveries ranged from 29.3 $\pm$ 4.5 % to 74.6 $\pm$ 2.2 % using an elution method based on sonication in elution buffer (1 % (w/v) beef extract, 50 mM glycine, pH 9.0). In another study, Botzenhart and Hock (2002) found that the highest efficiency of poliovirus elution from drinking water biofilms was obtained with an elution buffer containing skim milk instead of beef extract (0,1 % (w/v) skim milk, 0.375 % (w/v) glycine, pH 9.5). For the elution of bacteriophage PRD1, coliphage MS2 and coliphage φX174 from fresh water sediments, a combination of shaking and sonication in elution buffer (10 % (w/v) beef extract, pH 9.0) yielded average recovery rates of 89 %, 92 % and 88 %, respectively (Skraber et al., 2009b).

In this work, the protocol based on sonication in an alkaline elution buffer supplied with 1 % (w/v) beef extract and 0.375 % (w/v) was most efficient to elute viruses from River Ruhr sediment and thus was used for the isolation of viruses in this field study.

6.1.1.2 Distribution of *E. coli* and somatic coliphages in river water, epilithic biofilms and sediments

Within this study, *E. coli* and somatic coliphages were frequently detected in surface water, epilithic biofilms and sediments of the River Ruhr. Moreover, it was found that the enhanced rainfall and increase in river flow rate coincided with a rise in concentrations of *E. coli* in the water by approximately 2.5 orders of magnitude, while no changes of *E. coli* concentrations were observed in epilithic biofilms and sediments. Apparently, biofilms and sediments were not influenced by short term variation of *E. coli* concentrations in the flowing water but rather maintained a basal level of these organisms.

In a previous monitoring study of the River Ruhr (Strathmann et al., 2016), a trend towards enhanced concentrations of *E. coli* and intestinal enterococci was observed with precipitation on the day of sampling and two days before. Garcia-Aljaro et al. (2017) observed an increase in the number of *E. coli* in a Spanish river after heavy rainfall. The majority of *E. coli* concentration peaks of a Canadian river were linked to combined sewer overflow discharges that were caused by precipitation exceeding 10 mm or spring snowmelt (Madoux-Humery et al., 2016).

In the current study, the significant increase in *E. coli* levels in the bulk water was not reflected by the relatively constant concentrations inside the biofilms, indicating that no substantial attachment of planktonic *E. coli* to epilithic biofilms and sediments had occurred during the period of strong precipitation. Similarly, Hirotani and Yoshino (2010) reported that increased densities of *E. coli* in a Japanese river were not followed by increased concentrations in natural biofilms on pebbles. These observations suggest that the ratio of *E. coli* in bulk water and biofilms may vary in situations, where a substantial increase in the input of fecal organisms such as discharges of raw sewage occurs (Devane et al., 2014). During the period prior to the increased precipitation, an accumulation of *E. coli* in the epilithic biofilms and sediments was observed with three orders of magnitude higher densities compared to the flowing water, while this difference was not apparent after the precipitation event. The reason may be the rainfall-mediated discharge of fecal organisms into the river with concomitant increase in *E. coli* concentrations in the water column. In general, the occurrence and sometimes the accumulation of *E. coli* in river sediments have been described in a number of studies of freshwater streams in various geographic regions (Balzer et al., 2010; Byappanahalli et al., 2003; Devane et al., 2014; Garcia-Aljaro et al., 2017; Muirhead et al., 2004; Obiri-Danso and Jones, 1999). Several mechanisms may underlie the presence of

E. coli in biofilms of fecally polluted rivers such as the input from external sources (e.g. wastewater treatment plant effluents, combined sewer overflows, runoff from agricultural areas, wildlife), transport and incorporation into biofilms as well as the adoption of survival strategies to persist in the competitive environment of aquatic biofilms (Byappanahalli et al., 2003; Marino and Gannon, 1991). Besides, a positive correlation between the water temperature and the presence of *E. coli* in biofilms was observed by Hirotani and Yoshino (2010), the authors suggested that this could be attributed to growth of *E. coli* in the biofilm. Literature data indicate that river sediments and epilithic biofilms can temporarily harbor different bacterial and protozoan enteric pathogens including *Campylobacter*, *Salmonella*, *E. coli* O157 and antibiotic-resistant *E. coli*, *Cryptosporidium* and *Giardia* (Devane et al., 2014; Garcia-Aljaro et al., 2017; Maal-Bared et al., 2013; Subirats et al., 2017).

In contrast to fecal indicator bacteria like *E. coli*, coliphages, which are also of fecal origin, share many properties with human enteric viruses such as morphology, structure and mode of replication. For this reason, coliphages were proposed as indicators for fecal pollution to assess the behavior of enteric viruses in water environments (Brezina and Baldini, 2008; Contreras-Coll et al., 2002; Jiang and Chu, 2004; WHO, 2017) and could be considered for inclusion in verification and surveillance monitoring where source waters are known to be affected by human fecal waste (WHO, 2017). However, there is no direct correlation between numbers of coliphages and enteric viruses and thus, somatic coliphages cannot be absolutely relied upon as an indicator for enteric viruses in surface waters (Hot et al., 2003; Jiang and Chu, 2004; Jurzik et al., 2010). Somatic coliphages were detected in most of the water, biofilm and sediment samples from the River Ruhr, however a high temporal variation of coliphage concentrations in water throughout the study period as it was found for *E. coli* was not observed. The mean concentration of coliphages in water was significantly higher in the period after enhanced rainfall, whereas there was no significant difference in concentrations found in biofilms and sediments before and after rainfall. Throughout the study period, the concentrations of somatic coliphages in epilithic biofilms and sediments were elevated by up to four orders of magnitude compared to those in the flowing water. This indicates that coliphages have a higher tendency to accumulate in biofilms on stones and sediments in the riverbed compared to *E. coli* and/or are more persistent in biofilms and sediments than *E. coli*. The observed accumulation of coliphages in biofilms is consistent with data from the

literature showing that coliphages may attach to and accumulate in biofilms found in river environments (Devane et al., 2014; Garcia-Aljaro et al., 2017).

The present study demonstrated the co-existence of somatic coliphages and their potential host cells *E. coli* both in the water column and in epilithic biofilms as well as sediments. Previous laboratory studies showed that replication of low-temperature coliphages isolated from river water at temperatures of approximately 20 °C is generally possible (Seeley and Primrose, 1980). In this work, propagation of somatic coliphages in *E. coli* monospecies biofilms was not observed under simulated environmental conditions, as discussed later (6.2.2.2).

6.1.1.3 Distribution of enteric viruses in river water, biofilms and sediments

Different enteric viruses are present in anthropogenically influenced surface waters that receive effluents from wastewater treatment plants (Fong et al., 2010; Hamza et al., 2011a). In this study, several enteric viruses (HAdV, EV, RoV, NoV GII) were detected in the River Ruhr with HAdV being most dominant with 21 out of 24 water samples positive in qPCR. Since qPCR alone does not give information about infectivity of these, cell culture-based methods were used, showing that 15 of 24 samples were positive for HAdV. The high prevalence for HAdV in surface water compared to other viruses concurred with studies from the literature (Albinana-Gimenez et al., 2009; Hamza et al., 2009; Haramoto et al., 2010; Prevost et al., 2016), showing that HAdV is found with a high prevalence in different aquatic systems all over the world. Additionally, human derived enterovirus, group A rotavirus and norovirus GII were detected in this study via both qPCR and cell culture, which indicates further that other enteric viruses with human fecal origin are present in the River Ruhr. In this work, the virus prevalence of enteric viruses in water was HAdV>EV>RoV>NoV GII, while the prevalence in biofilms and sediments was similar (HAdV>EV>RoV, NoV GII). The data obtained here are in consistence with Hamza et al. (2009), who found a large panel of enteric viruses in the River Ruhr. Regarding epilithic biofilms and sediments, only qualitative detection of human enteric viruses using qPCR was possible. It should be mentioned that the efficient detection of viruses in complex samples such as surface water, biofilms or sediments can be affected by various environmental and methodological parameters. For instance, during concentration of viruses from large volumes of water or elution from biofilms or sediments, inhibitory substances can be enriched that might interfere with downstream analysis. Moreover, different efficiencies for the extraction of different viral DNA or RNA

molecules using the same extraction kit causes uncertainties in the overall process. The relatively high LOQ using qPCR for virus quantification in epilithic biofilms and sediments was caused by the fact that sample sizes were significantly smaller than for water samples which does not allow a sufficient pre-concentration of viruses from the sample matrix. Therefore, it is likely that the low detection rates severely underestimate the true occurrence of enteric viruses in the samples. Attempts to lower the high LOQ by virus propagation using cell culture and subsequent qPCR were unsuccessful due to the high load of bacteria and other particles in the samples interfering with the cell lines used (data not shown). Thus, it was decided to assess the presence of enteric viruses in biofilms and sediments qualitatively.

Nevertheless, approximately half of the biofilm and sediment samples were positive for HAdV DNA, one sample for each was positive for human enterovirus RNA. Although it was not possible to elucidate the distribution of enteric viruses between water, biofilms and sediments, this study demonstrated that infectious human enteric viruses are present in surface water of the River Ruhr and that potentially infectious enteric viruses could be detected in biofilms and sediments using molecular methods. It remains unclear whether enteric viruses accumulated to the same extent as somatic coliphages and consequently if coliphages can be used as surrogates for enteric viruses in river biofilm environments. In a similar study, HAdV and group A rotavirus were detected in sediments in a river system in central Brazil (Elmahdy et al., 2016), however the concentrations detected in water were much higher than in our study, most likely due to the different geographical area, insufficient water treatment, non-point fecal sources and the use of a different set of primers for qPCR analysis of viruses. In addition, the authors found that HAdV concentrations were elevated by three orders of magnitude compared to the overlying water. Moreover, enterovirus was detected in river sediments in other studies (Ali et al., 2004; Garcia-Aljaro et al., 2017). This might have an impact on human health because sediment-bound viruses were shown to have a lower inactivation rate in batch experiments compared to viruses in the flowing water (Chung and Sobsey, 1993; Sakoda et al., 1997).

6.1.1.5 Correlation among environmental and microbial parameters

A significant correlation between the flow rate and the concentration of *E. coli* and coliphages in water was found, indicating that the fecal pollution and thus the load of bacteria and coliphages increased with increasing flow rate. Besides these indicator organisms, the concentration of HAdV also increased with increasing flow rate showing that not only coliphages but also human enteric viruses were introduced into the river. The concentrations of both HAdV and coliphages correlated significantly. The concentration of coliphages in water also correlated with the concentration of *E. coli* in water, which is in concurrence with Brezina and Baldini (2008). It was proposed that the correlation between enteric viruses and indicator bacteria might be dependent on the age of the fecal pollution (Jiang and Chu, 2004). Additionally, Hot et al. (2003) reported that enterovirus RNA could be used as reliable indicator for pollution with enteric viruses, whereas the detection of indicator bacteria or coliphages is not sufficient.

According to results obtained in this study, variations of neither *E. coli* nor coliphage concentrations in water over time are linked to concentrations in the riverbed. While no significant correlation between the concentrations in water and biofilms or sediments was found in this work, Hirotani et al. (2013) found a positive correlation between coliphage concentrations in water and biofilms, whereas this was not the case for *E. coli* concentrations.

Indeed, the flow rate of the River Ruhr and the discharge of fecally polluted water have an influence on the water quality and thus should be monitored to estimate the hygienic risk for bathing in urban rivers in densely populated areas such as the Ruhr. Potential pollution sources of the River Ruhr including the stretch of the river and the barrier lake (Lake Baldeney) covered in the present study are mainly discharges from sewage treatment plants and additionally by discharges from combined sewer overflows after strong rainfall events, while input from diffuse sources have a minor impact (Strathmann et al., 2016). Additionally, this work demonstrated that the concentrations of *E. coli* and coliphages correlated with HAdV in water, even though it is not clear whether that also applies for virus retention in epilithic biofilms and sediments.

6.1.1.6 Conclusion

This study shows that epilithic biofilms and sediments of the urban River Ruhr (Germany) present a reservoir not only of fecal indicators (*E. coli*, somatic coliphages), but also of human pathogens as demonstrated here for enteric viruses. Elevated concentrations of *E. coli* and somatic coliphages in epilithic biofilms and sediments compared to the flowing water suggest retention and persistence of these indicators in the riverbed. Enhanced precipitation in combination with an increased flow rate were associated with elevated levels of the fecal indicators in the river water, probably originating mainly from higher upstream discharges from wastewater treatment plants and combined sewer overflow events. From a health perspective, contamination with fecal pathogens poses a risk to human health in surface waters used for recreational activities such as swimming, and results in the deterioration of microbiological quality of raw water abstracted for drinking production. This study confirms the notion that river biofilms and sediments can be important reservoirs of microbial pathogens and may contribute to a public health risk, in particular due to the mobilization of pathogens from the riverbed during sediment-disturbing events such as floods or extreme precipitation events.

6.1.2 Detection of viruses in drinking water systems for animals

In this field study, drinking water systems of five different piglet breeding farms were analyzed for the occurrence of somatic coliphages and animal viruses that are relevant in pig farming. One aim was to determine, whether coliphages or animal pathogenic viruses would be found in biofilms from pipe surfaces of the distribution system, where they could be retained during flushing of the system. This type of field study was chosen in the context of this work because drinking water for animals are technical water systems and were assumed to have a higher probability of fecal contamination compared to drinking water systems for humans. It should be mentioned that drinking water systems for humans and animals are not fully comparable because systems for animals are not designed and built according to the guidelines that exist for drinking water systems for humans. Drinking water systems are required to provide appropriate amounts of water to the livestock. However, while quality and surveillance of drinking water for humans in Germany is strictly regulated by the Drinking Water Ordinance (TrinkwV, 2001), no such regulation exists about construction and maintenance of drinking water systems for animals. Although a publication about water quality requirements in animal farming addresses several technical, chemical and

microbiological aspects, the authors concluded that further research is needed in this field (Kamphues et al., 2007). Especially in large-scale animal farms, individuals are often in close contact between each other, resulting in increased exposure to fecal matter and facilitated transmission of pathogens between individual animals. Although studies have investigated the occurrence of relevant viruses, including porcine adenoviruses, porcine circoviruses, and hepatitis E viruses in infected animals, feces and slurries form pig farms (Derbyshire and Brown, 1978; Vasickova et al., 2009; Yang et al., 2003; Zhou et al., 2016), the potential role of contaminated drinking water is widely unknown. While stables are cleaned and disinfected on a regular basis, drinking water systems might be neglected, although they could harbor pathogens which were introduced by retrograde fecal contamination. Porcine viruses can cause severe illnesses of the animals and consequently high economical damage to farmers. Moreover, hepatitis E virus is known as a zoonotic virus that could potentially be transmitted from pig to human (Meng et al., 1998), which underlines the importance for human health to control viral pathogens in animal farming.

6.1.2.1 Evaluation of the virus concentration method

In this field study, viruses were concentrated from water and biofilms samples using a method based on PEG precipitation. In order to assess the suitability of the selected method for the concentration of viruses from water samples, recovery experiments were performed. The three test viruses that were available in the laboratory during this work (HAdV, MNV and coliphage φX174) were spiked to 45 ml of deionized water, concentrated using the PEG concentration method and subsequently quantified by qPCR. The average recovery rates were 38 $\pm$ 17 % for HAdV, 12 $\pm$ 6 % for MNV and 43 $\pm$ 18 % for coliphage φX174, using qPCR, respectively. Apparently, the RNA virus MNV showed the lowest recovery with the concentration method used. Besides qPCR, the recovery of infectious coliphages was also determined via plaque assay, results were comparable to the results obtained by qPCR (40 $\pm$ 14 %). It should be mentioned that deionized water might not adequately reflect biofilm samples from this field study. Moreover, biofilm biomass, consistency and composition varied tremendously between different samples (data not shown), which implies that the recovery of viruses might have been different between samples. Other studies have shown that the PEG concentration method is suitable as first or second concentration step for a variety of environmental water samples, including tap water (El-Senousy et al., 2013), irrigation water (El-Senousy et al., 2013), raw and treated wastewater (Amdiouni et al., 2012;

Hmaïed et al., 2016), with significant differences in virus recovery rates. For instance, Amdiouni et al. (2012) reported a high recovery (78.5 ± 11.0 %) of viruses from wastewater samples, while El-Senousy et al. (2013) found lower recovery rates of 28.0 to 48.0 % for irrigation water. Although virus recovery rates differ between different sample types and volumes, this method was chosen in this work because it is cost-effective, requires only modest skills (Matrajt et al., 2018) and is widely used to concentrate viruses in complex water samples.

6.1.2.2 Occurrence of viruses in water and biofilms from drinking water systems for animals

After the concentration method was evaluated, 25 water and 59 biofilm samples from five different piglet breeding farms in Germany were concentrated and subsequently analyzed using cultural and molecular biology methods. Somatic coliphages were quantified using the plaque assay, animal pathogenic viruses were analyzed using qPCR. During the sampling period, somatic coliphages were detected in several samples from different piglet breeding farms, however the overall frequency of detection was higher in biofilms than in water. For each sampling day and farm, one sample was taken from the inflowing water, which – in all cases – did not contain somatic coliphages. This finding was expected because the inflowing water was drinking water in most cases, while only some farms used well water to supply animals (data not shown). It can be assumed that the contamination with coliphages occurred in the stable itself, potentially by retrograde contamination with fecal matter from the animals. The higher abundance of coliphages in pipe biofilms suggests that these biofilms could act as a reservoir for phages and thus lead to a recontamination of the water when continuous release of viruses or detachment of biofilm material occurs. Besides coliphages, samples were also analyzed for selected animal viruses. A total number of 16 samples was positive for PAdV, using qPCR as a detection method. While only one water sample was positive, 15 biofilm samples showed positive PAdV signals. For PAdV in biofilms, the highest frequency of detection was found at piglet farm 3 (7/8 positive biofilm samples), interestingly the high prevalence was not found for somatic coliphages in this farm.

The second - more frequently detected - animal virus was HEV. In this study, 61 % of all samples were positive for HEV, using qPCR. Interestingly, the frequency of detection was often higher in water than in biofilms, also virus concentrations were much higher than for PAdV or somatic coliphages. As for PAdV, no detection of infectious HEV was conducted,

but viral RNA was analyzed. However, it is likely that results for HEV were false-positive, because it turned out that the qPCR assay was not specific for HEV. Several negative controls (for virus concentration, RNA extraction, qPCR) showed positive results in qPCR (data not shown). A contamination with HEV RNA during sample processing was assumed to be unlikely because positive results occurred sporadically without any trends that suggested a contamination in a certain step of sample preparation. Moreover, several samples from inflowing waters were tested positive. On the contrary, other viruses were not detected in the inflowing water, thus it is not plausible that the samples were HEV positive. When analyzing the PCR products for HEV by agarose gel electrophoresis, more than one band was observed for samples and negative controls. This indicates that at least the selected primers did not allow a specific amplification. At this point, it is unknown whether the hydrolysis probe used for HEV was unspecific, too. The primer pair and probe used were designed by Jothikumar et al. (2006), being applicable for detection of genogroups 1-4. Although authors tested the specificity of the assay with several human viruses, they did not test complex environmental samples with high loads of foreign DNA for unspecific amplification. Therefore, it is not valid to trust the positive qPCR results for HEV, but further research is needed to verify the high occurrence of HEV. In a recent review, Salines et al. (2017) found that HEV prevalence varies between different studies and also between farms within the same study, suggesting that several factors are relevant for infection of pigs by HEV. On the basis of this study, it is unknown whether piglets from the farms were infected with HEV or not.

The two other viruses considered in this work, PRRSV and PCV2, were not detected in any of the water or biofilm samples from this study. This result can either be explained if the viruses did not adsorb to biofilms in the drinking water distribution system, or these two viruses did not circulate in the stables tested. Although there are no data for infected animals, the latter is the most likely explanation, because PRRSV and PCV2 cause severe symptoms in piglets, which would probably have been noticed by the farmers. Supporting this hypothesis, a previous study has shown that the prevalence of PCV2 is relatively low in pig farms in southern Germany (Eddicks et al., 2016).

In conclusion, this field study showed that drinking water systems for animals in this study were contaminated with viruses, although their hygienic relevance in transmission is not known. In addition, biofilms in the distribution system can act as reservoirs for somatic coliphages and animal pathogenic viruses, showing that also biofilms in technical water systems should be considered for virus retention. It is worth to mention that animal viruses

were detected using qPCR, positive qPCR results do not necessarily indicate a potential health risk, because only viral DNA or RNA is detected instead of infectious virus particles. Thus, it is not possible to draw conclusions on the relevance for animal health from the results of this work. As far as is known, this is the first study showing that somatic coliphages and porcine viruses occur in water and biofilms from drinking water systems of piglet breeding farms. To date, there are no literature data to compare the abundance of viruses found in this work with other studies.

6.2 Laboratory experiments

6.2.1 Interaction of enteric viruses and somatic coliphages with drinking water biofilms

In this part, the interaction between three selected viruses (HAdV, MNV, coliphage φX174) and drinking water biofilms was investigated in laboratory experiments. HAdV as DNA virus was chosen in this context because it was found to be associated with waterborne outbreaks of viral gastroenteritis and has a high prevalence in the urban water cycle (Albinana-Gimenez et al., 2009; Hamza et al., 2009; Haramoto et al., 2010; Prevost et al., 2016). MNV was used as surrogate for human noroviruses, which represent RNA viruses that cause waterborne outbreaks worldwide (Blanco et al., 2017; Friedman et al., 2005; Kauppinen et al., 2017). While no appropriate cell line exists for the quantification of infectious human noroviruses to date, MNV can be quantified using the murine macrophage cell line RAW 264.7 (Wobus et al., 2006). Coliphage φX174 was included in this study because it belongs to the group of somatic coliphages, which are discussed as potential indicator for enteric viruses in the urban water cycle, as reviewed by Jofre et al. (2016). To study the potential of drinking water biofilms to retain viruses, artificial drinking water biofilms were grown in stainless steel reactors for 14 d. Subsequently, the three viruses were added to the water phase and reactors were further incubated for 7 d under stagnant or flow conditions. The concentration of viruses in water was assessed at different time points (0 d, 1 d, 7 d), the concentration of viruses in biofilms was analyzed after 1 d and 7 d of incubation. Finally, the distribution of viruses was determined by comparing the concentration in water and biofilms.

6.2.1.1 Drinking water biofilms as reservoirs for pathogens

In order to elucidate the potential of drinking water biofilms to retain viruses, drinking water biofilms had to be grown in the laboratory. For this purpose, coupons of the elastomeric material EPDM were chosen to generate biofilms within a relatively short period of time. In a previous study comparing different materials for the formation of drinking water biofilms, the authors found that biofilms on EPDM surfaces had higher total cell counts and heterotrophic plate counts within 14 d compared with two types of polyethylene or copper (Moritz et al., 2010). Moreover, EPDM coupons were successfully used in combination with stainless steel reactors used in this work in order to study the incorporation and persistence of facultative pathogenic bacteria (*L. pneumophila* and *P. aeruginosa*) into drinking water biofilms (Michalowski, 2012; Moritz et al., 2010). Therefore, the same reactor setup was used to study the interaction of viruses with drinking water biofilms in this study.

Within this work, macroscopically visible biofilms with average total cell counts of $2.3 \times 10^8 \pm 6.6 \times 10^7$ cells/cm^2 developed on the surfaces of the coupons, the heterotrophic plate count was approximately one order of magnitude lower, which is in accordance to a previous study by Bressler et al. (2009), who found similar total cell counts using the same material in a similar setup for biofilm growth. On the other hand, Moritz et al. (2010) found lower total cell counts of approximately 1.0×10^7 cells/cm^2 after 14 d of growth on EPDM, using the same experimental setup. In a field study, Kilb et al. (2003) reported high total cell counts between 8.6×10^6 cells/cm^2 and 1.8×10^9 cells/cm^2 in biofilms from EPDM-coated valves in drinking water distribution systems, whereas biofilms from nitrile butadiene rubber (NBR)-coated valves revealed lower cell counts. These data show that EPDM is an appropriate material for simulated growth of drinking water biofilms in the laboratory. The extensive biofilm formation can be explained because materials such as EPDM potentially leach nutrients, which in turn promote bacterial growth (Mao et al., 2018; Schaule et al., 2007). In addition to the EPDM, also the tubing materials which were used to connect inlet and outlet to the reactors probably contributed to the nutrient availability in the water. The Tygon® tubes used in this work could also have facilitated leaching of nutrients to the water, which was confirmed by extensive biofilm growth not only on EPDM, but also inner surfaces of the tubes.

In this work, different flow conditions were applied after the inoculation of viruses. While total cell counts of biofilms were higher after incubation under stagnant conditions compared to flow conditions, no difference was observed comparing heterotrophic plate counts.

Consequently, the percentage of culturable heterotrophic bacteria was higher under flow conditions. Moreover, the percentage of culturable cells increased from 9.2 ± 0.0 % after 1 d of incubation to 14.1 ± 0.3 % after 7 d of incubation under flow conditions, whereas it decreased under stagnant conditions (from 6.4 ± 1.8 % after 1 d to 1.8 ± 0.9 % after 7 d of incubation). This could be explained by the recirculation of nutrients in the system under flow conditions or by reduced culturability of bacteria due to oxygen depletion under stagnant conditions.

In this study, the experiments were conducted in different seasons of the year. Although the reactor setup was the same for all experiments, it turned out that the amount of biofilm that grew on the coupons differed substantially between individual experiments, which was observed macroscopically, but also by differences in wet weight, total cell count and heterotrophic plate count. Interestingly, the variation between different experiments was higher than the variation between two reactors running in parallel with the same inflowing water (data not shown). It it is possible that the microbial communities, EPS composition and biofilm structures differed significantly between experiments, however this was not assessed in this work. It is known that the community composition of biofilms in drinking water depends on many parameters, including the type of source water, temperature, pH, water hardness and flow conditions (Douterelo et al., 2016; Douterelo et al., 2017; Fish et al., 2017; Kelly et al., 2014; Revetta et al., 2016). While most of these parameters are relatively constant in the drinking water throughout the year, the water temperature varied between 12.1 °C and 21.9 °C within this work, which could also have influenced the growth of biofilms. Although the water temperature is known to significantly affect the extent of biofilm formation (Liu et al., 2016), seasonal differences can also be expected in drinking water distribution systems, therefore the selected laboratory system was assumed to reflect a realistic scenario.

6.2.1.2 Decrease of virus concentrations in drinking water

After the addition of viruses to the biofilm reactors, water samples were taken to determine the concentration of the respective viruses at time points 0 d, 1 d and 7 d. The concentrations of viruses in water decreased over time in most experiments using qPCR and culture-dependent quantification. However, for some experiments, the concentration of all three target viruses was equal or higher one day after the inoculation than at the beginning of the experiment, using qPCR. The absence of host cells for reproduction of enteric viruses or

coliphages implies that there was no propagation during the incubation period. Instead, it is possible that viruses could have been present in aggregates in the beginning of the experiments, which were disrupted during the experiment. Young and Sharp (1977) showed that a large proportion of polioviruses occur as aggregates when released from infected host cells, which are not effectively disrupted by simple dilution in water. Since the viruses were added as stock suspensions to the reactors, this could also have introduced viral aggregates into the system. Aggregation was described for a variety of viruses and can be induced by changes in pH, salinity, presence of cations, natural organic matter or polyelectrolytes, which can also influence the behavior and stability in the aquatic environment (Gerba and Betancourt, 2017; Pinon and Vialette, 2018). Besides, aggregation can potentially cause underestimation of virus concentrations using quantification methods such as cell culture or plaque assay (Hassard et al., 2016; Langlet et al., 2007). Therefore, virus concentrations were assessed using both qPCR and cultural methods in this work.

In order to compare the decrease of virus concentrations in water, virus reduction rates were calculated based on concentrations at time points 1 d and 7 d, assuming a linear decrease in this period. On average, virus concentrations in water determined by qPCR decreased by 1-1.5 log units after 7 d of incubation, regardless of the flow conditions applied. Comparing the reduction rates based on qPCR data, HAdV showed the highest reduction between days 1 and 7 (0.20 ± 0.09 log/d), followed by MNV (0.16 ± 0.16 log/d) and coliphage φX174 (0.13 ± 0.13 log/d). Using cultural methods, the concentration of HAdV and coliphage φX174 in water decreased even faster, while no decrease was observed for MNV, using cell culture (as discussed later). On average, the concentration of infectious HAdV decreased by 1-3 log units within the first day of incubation, whereas it decreased by 1-3 log units within 7 d for coliphage φX174. Consequently, the reduction rates for HAdV and φX174 were higher based on data from cultural quantification than on data from qPCR. Especially the decrease of the HAdV concentration was high when reactors were incubated under flow conditions, no infectious HAdV were detected in water after 7 d of incubation, using cell culture. Thus, it was not possible to calculate the reduction rate for flow conditions. Instead, it was only calculated for stagnant conditions, resulting in a reduction rate of 0.18 ± 0.13 log/d. The concentration of infectious coliphage φX174 in water decreased by 0.34 ± 0.12 log units per day, but the decrease under stagnant conditions was lower than for HAdV (data not shown). The faster reduction of the concentration of infectious viruses compared to virus concentrations determined using qPCR could be explained because both viral particles that

lost their infectivity and free viral DNA or RNA would still be detected by qPCR, whereas culture-based methods only detect viruses that are capable of infection (Hamza et al., 2011b).

In this study, no clear change of the concentration was observed for infectious MNV during the incubation period, using detection by cell culture. Thus, the reduction rate for infectious MNV was lower than the reduction of MNV genome concentration, which is assumed to be very unlikely. In fact, it is more probable that the quantification using cell culture was not correct. The quantification of MNV via cell culture is based on end-point dilution of the sample and subsequent statistical analysis of the observed effects on RAW 264.7 cells. However, cytopathic effects are evaluated by microscopic analysis, which were - in case of RAW 264.7 cells - not clearly visible for water and biofilm samples from this work. Cells did not show detachment from the bottom of tissue culture plates, but only morphological changes were found based on the sample dilution and regarded as “early cytopathic effect”. However, water and biofilm samples without the addition of MNV caused similar effects in the assay (data not shown). Thus, it is likely that some wells were rated positive although they did not contain infectious MNV, which caused overestimation of the calculated $TCID_{50}$. At this point, it remains unclear why the cell line RAW 264.7 reacted differently when exposed to water and biofilm samples compared to pure MNV from virus stocks. Interestingly, Agnihothram et al. (2015) described that co-infection of RAW 264.7 cells with *Salmonella enterica* can interfere with MNV infection and subsequent virus-induced apoptosis, which indicates that bacteria in the sample can potentially impede the quantification of infectious viruses. Although samples from this work were centrifuged prior to analysis to reduce the concentration of bacteria, it is possible that remaining microorganisms or chemical substances in water and biofilm samples caused false positive effects. However, there is still a gap of knowledge about factors that can influence the assay using different types of samples. It is worth to mention that the quantification of HAdV by A549 cells did not cause similar problems. Instead, cytopathic effects were easily visible because cells detached from the bottom of the tissue culture plates. In contrast to RAW 264.7 cells, no false-positive effects were observed in A549 cell cultures for water or biofilm samples without HAdV.

The reduction of virus concentrations in water found in this work reflect the sum of three different processes: First, viruses can be inactivated by biological or physicochemical stressors, including elevated temperature (Elmahdy et al., 2018; Hurst et al., 1980; Lo et al., 1976; Nasser et al., 1993; Yates et al., 1985). For instance, Lo et al. (1976) demonstrated that the stability of poliovirus 1, echovirus 6 and coxsackievirus B-5 in artificial seawater

decreased with increasing water temperature (4 °C < 15 °C < 25 °C). Similarly, temperatures of 30 °C were found to cause faster inactivation of hepatitis A virus and poliovirus 1 in groundwater compared to 10 °C (Nasser et al., 1993). Besides temperature, also other stressors such as shear forces (Thompson et al., 1998) or the excretion of proteolytic enzymes by bacteria (Nasser et al., 2002; Ward et al., 1986) can lead to virus inactivation. Gordon and Toze (2003) studied the stability of coliphage MS2, poliovirus and coxsackievirus in groundwater and observed an increased virus inactivation in aerobic waters. The authors concluded that the increased activity of aerobic bacteria presumably enhanced the inactivation of viruses. There are several more factors that can affect the stability of viruses in aquatic environments, including acidic or alkaline pH (Feng et al., 2003; Shahid et al., 2009), presence of OH^- radicals (Shahid et al., 2009), Fenton-like processes (Nieto-Juarez and Kohn, 2013) or sunlight (Elmahdy et al., 2018; Love et al., 2010; Sinton et al., 2002), however these are assumed to be negligible in this study because experiments were performed in the dark using stainless steel reactors.

Besides inactivation, viruses can adsorb to abiotic surfaces, in this case to the walls of the stainless steel reactors or tubing material and tube connectors. More importantly, the virus concentration in water can potentially decrease as a consequence of adsorption to the biofilms that were present on tubing and EPDM.

Comparing the obtained reduction rates for the three target viruses to the literature, values from this work appeared to be relatively high. However, the difference between reduction rates from this work and values from the literature could be attributed to the experimental system used. While this study focused on the adsorption of viruses to previously grown drinking water biofilms, most of the authors did not explicitly grow biofilms. Instead, they determined virus stability in aquatic systems using microcosms without considering adsorption to biofilms, which mainly assessed virus inactivation in water.

For instance, Ogorzaly et al. (2010) inoculated groundwater in glass bottles with viruses and reported a decay rate of 0.0036 log/d for HAdV2 genomes at 20 °C. Similarly, Kauppinen et al. (2017) recently reported a reduction rate of 0.0022 log/d for NoV genomes and 0.0014 for HAdV genomes in drinking water at 4 °C. Also for infectious viruses, authors found smaller reduction rates compared to this work. Lee and Sobsey (2011) reported an inactivation rate of 0.04 log units per week for infectious coliphage φX174 in ultrapure water supplemented with 10 % PBS at 25 °C. In another study, authors spiked HAdV to tap water and found that the

concentration of infectious viruses decreased by 0.3 log units within the first seven days, which equals a reduction rate of 0.04 log/d (Sun et al., 2016). Similarly, Elmahdy et al. (2018) determined comparable inactivation rates for infectious recombinant adenovirus (0.04 log/d) and MNV (0.06 log/d) in surface water.

Apparently, it is not possible to compare virus reduction rates in water between this work and most studies from the literature. To date, there is a lack of data from studies that included the adsorption of viruses to aquatic biofilms when assessing the concentration in water. In one study, Helmi et al. (2008) spiked poliovirus, coliphage φX174 and coliphage MS2 to a rotating annular reactor with previously grown drinking water biofilms at 10 °C. Authors monitored the concentration of the target viruses in water using cultural methods for 48 h after spiking. The concentrations at the beginning of the experiment and after 48 h of incubation are reported and correspond to reduction rates of 0.14 log/d, 1.05 log/d and 1.68 log/d for poliovirus, coliphage φX174 and coliphage MS2, respectively. Interestingly, the authors also determined the decrease of virus concentration in drinking water without the presence of biofilms, which resulted in smaller reduction rates for the two coliphages. This shows that the adsorption of viruses to biofilms significantly contributes to the reduction of the virus concentration in water. Comparing the reduction rates to the rates reported in other studies, the work by Helmi et al. (2008) indicates that virus reduction rates are substantially higher when biofilms are present in the experimental setup, as it was also found in this work. Moreover, the reduction rates in water apparently vary substantially between different viruses, potentially due to different virus stabilities, adsorption rates and different experimental setups.

In conclusion, the decrease of virus concentrations in drinking water from the experiments of this work suggest that viruses were not only inactivated, but may also have adsorbed to the drinking water biofilms under all conditions tested.

6.2.1.3 Accumulation of viruses in drinking water biofilms

One major objective of this work was to determine the potential retention of viruses in drinking water biofilms. Although drinking water biofilms can be found ubiquitously in water storage tanks or drinking water distribution systems, a relevance for human health would require a permanent or temporary contamination of the water with viruses, because enteric viruses cannot propagate in the distribution system in the absence of host cells. According to the German drinking water ordinance (TrinkwV, 2001), drinking water must not contain

pathogens, infectious enteric viruses could only enter the distribution network in rare cases of severe treatment or disinfection failure, repair works or leakages in the distribution network and subsequent contamination from outside. Once a fecal contamination had occurred, biofilms could potentially retain pathogens including enteric viruses, which could be released to the water again after a certain time. Thus, drinking water biofilms could potentially act as reservoir for viruses, even after the cause of the contamination was rectified. Supporting this hypothesis, Kauppinen et al. (2012) found that HAdV persisted in drinking water biofilms of a simulated distribution network that was subjected to a fecal contamination, whereas the bacterial indicator *E. coli* was no longer detected after the network containing contaminated water was flushed. Besides, the retention of viruses in drinking water biofilms could be of major hygienic relevance, because viruses that adsorbed to biofilms or particles were found to have a higher resistance towards chlorination than viruses in the bulk water (Hejkal et al., 1979; Hejkal et al., 1981; Mazaheritehrani et al., 2014; Quignon et al., 1997a; Quignon et al., 1997b).

In this work, samples from water and biofilms were taken after 1 d and 7 d to assess the distribution of viruses at these timepoints. Different environmental conditions were simulated to mimic a typical drinking water distribution system in Germany. On the one hand, different hydraulic conditions were compared by either incubating reactors under stagnation or circular flow, on the other hand the influence of water hardness was tested under stagnant conditions. The concentration of Ca^{2+} in the water can influence the composition and mechanical stability of biofilms (Kelly et al., 2014; Korstgens et al., 2001; Shen et al., 2018), moreover it could potentially affect the adsorption of viruses to biofilms due to the generation of positive charges in the EPS matrix.

Using qPCR for the quantification of viruses, most experiments showed an accumulation of HAdV, MNV and coliphage φX174 in the biofilm compared to water by 1-3 log units, with high variances between individual experimental replicates. While the concentration of viruses in biofilms was higher than in water for most experiments, two (out of six) did not show this effect in case of MNV. With this exception, no clear differences concerning the distribution in water and biofilms were observed between the three viruses. In many cases, the ratio between the virus concentration in the biofilm and the virus concentration in water increased from 1 d to 7 d of incubation, showing that viruses were further enriched in the biofilm within this period or concentrations were faster reduced in water than in biofilms. On average, lower accumulation was found under circular flow conditions compared to stagnant conditions,

which indicates that virus retention depends on the hydraulic conditions. This also implies that viruses could be released from the biofilm when conditions change from stagnant to flow conditions. However, this finding should be verified in further experiments due to high variation between replicates in this work.

Data based on qPCR alone do not allow any statements about health significance, because it is not possible to distinguish between infectious and non-infectious viruses. Therefore, viruses were also quantified using the plaque assay for coliphage φX174 and cell culture for HAdV and MNV. In general, qPCR results were confirmed by the cultivation-based methods, showing that not only viral DNA/RNA, but also infectious viruses tend to adsorb to drinking water biofilms. As found with qPCR, concentrations determined by cultural methods were 1-3 log units higher in biofilms compared to water after 1 d and 7 d of incubation for most experiments. Variations between individual experiments were relatively high, whereas minor differences were observed between two reactors that were run in parallel. For water and biofilm samples, absolute concentrations determined by qPCR were higher than concentrations determined by cultural methods (data not shown). This was expected, because qPCR includes detection of infectious viruses, damaged viruses and free viral DNA/RNA, whereas cultural methods only quantify infectious viruses. Concerning the virus distribution between water and biofilms, no trends were observed when comparing molecular and cultural methods. For some datasets, it was not possible to determine the ratio of infectious viruses between biofilms and water, because at least one of the two corresponding samples showed a concentration value below the limit of detection. Although relatively high initial virus concentrations (~10^7 gen.eq./ml of each virus) were used for spiking in the experiments, the inactivation of viruses and small sample volumes could be a reason for this issue. Interestingly, a high accumulation effect was found for infectious HAdV in artificially hardened water which contained 4 mM Ca^{2+}, concentrations in biofilms were 2-3.5 log units higher than in water after 7 d of incubation, using cell culture. This finding will be discussed later in this chapter.

The results obtained for infectious MNV were particularly confusing, because results found using qPCR were not confirmed using cell culture. While qPCR data showed that the ratio between the virus concentration in biofilms and water increased from 1 d of incubation to 7 d of incubation for all viruses, this was not the case for infectious MNV. Instead, the concentration of infectious MNV was higher in biofilms by 1-3 log units compared to water for all samples, regardless of the incubation time or conditions. However, cell culture results

for MNV in water and biofilms are highly doubtful due to reasons described above (6.2.1.2). In future experiments, other methods such as integrated cell culture qPCR (ICC-qPCR) should be included to verify the accumulation of infectious MNV in drinking water biofilms. In conclusion, both methods showed that all three target viruses accumulated in drinking water biofilms within 1 d of incubation under all conditions tested, although data for infectious MNV require verification.

It should be noted that variation was relatively high when experiments were repeated with the same conditions, whereas biological duplicates run in parallel in separate reactors showed minor variation. In some cases, the ratio of the virus concentration in biofilm and water differed by more than one order of magnitude between biological replicates. This indicates that other parameters than hydraulic conditions and water hardness probably had an important impact on virus retention. The process of adsorption is also influenced by the composition of the adsorbate, in this case the biofilm. As described above (6.2.1.1), biofilms differed in respect of biomass, total cell count and heterotrophic plate count between experiments. The water temperature was similar for all experiments after the addition of viruses (data not shown), because reactors were all incubated at room temperature. Therefore, a direct influence of the water temperature on virus adsorption appears to be unlikely. However, the water temperature during biofilm growth (prior addition of viruses) might have affected the biofilm growth and -structure, and therefore also potential binding sites for viruses. Thus, each biofilm in this work should be regarded as an individual ecosystem with unique structure and composition, depending on the growth conditions during each experiment.

Besides, different biofilm compositions might also have an influence on the purification and detection of viruses from the sample. For instance, the results from qPCR are highly dependent on the amount and purity of the DNA or cDNA. In addition, all previous steps (biofilm sampling, DNA/RNA isolation, reverse transcription, etc.) add uncertainties to the overall variation. Because there are many factors with a potential influence on the result from the experiments, it was decided to show results from individual experiments rather than average values from biological replicates under the same condition. Apparently, the high variation does not allow exact predictions about the distribution of viruses in water and biofilms. It should also be noted that biofilms that grew in tubing of the reactors were tested positive for the target viruses using qPCR (data not shown), which means that also these biofilms contributed to the overall reduction of viruses in the water phase. Interestingly, the total number of viruses that were associated with biofilms on EPDM and tubing was

approximately in the same order of magnitude as the number of viruses that remained in the water (data not shown). However, it was impossible to compare the exact distribution of viruses in each phase, because the volume of water in the system was not determined and thus the total number of viruses in water could not be calculated.

To date, there is only a limited number of studies which investigated the occurrence, persistence or survival of viruses in drinking water biofilms. Lehtola et al. (2004) stained suspensions of drinking water biofilms with SYBR Green I and subsequently detected virus like particles (VLPs) using epifluorescence microscopy. During their study, the authors observed that the number of VLPs was higher in biofilms grown on PE pipes compared to copper pipes. However, a microscopic detection of VLPs in complex samples such as biofilms does not specifically encounter viruses and thus could include false-positive results, which makes it difficult to evaluate the significance for human health. Another group found that polioviruses tend to accumulate in drinking water biofilms by a factor of 2-10 in a pilot drinking water distribution system (Quignon et al., 1997a; Quignon et al., 1997b). The authors also found that the presence of clay can enhance the accumulation effect of viruses in biofilms (Quignon et al., 1997b). In several studies, Storey and Ashbolt reported that different bacteriophages, including coliphage MS2, coliphage φX174 and *Bacteroides fragilis* phage B40-8 adsorb to artificially grown drinking water biofilms within 24 h of incubation under stagnant conditions (Storey and Ashbolt, 2001; Storey and Ashbolt, 2003a; Storey and Ashbolt, 2003b). Similarly, Helmi et al. (2008) inoculated poliovirus type 1, coliphage MS2 and coliphage φX174 into rotating annular reactors with previously grown drinking water biofilms and found that all viruses attached to the biofilm within 1 h of incubation, coliphage MS2 showed the highest rate of attachment among the three viruses. It should be noted that most research groups mentioned above performed studies based on either qPCR or detection of surrogate viruses such as bacteriophages instead of infectious enteric viruses. However, it remains to be confirmed whether bacteriophages show a comparable behavior as infectious enteric viruses.

In general, viruses are biological nanoparticles that are expected to show colloidal behavior in a dispersion medium (Gerba, 1984), which also includes the adsorption to surfaces. Studies have shown that processes of virus adsorption to sand and soil particles differs depending on the type of virus (Chrysikopoulos and Aravantinou, 2014; Dowd et al., 1998). There is evidence that also the adsorption of viruses to drinking water biofilms depends on the hydrophobicity of the virus (Pelleieux et al., 2012). The same was proposed earlier by

Långmark et al. (2005), who studied the adsorption of fluorescent microspheres to artificial drinking water biofilms showing that hydrophilic spheres accumulated to a larger extent than hydrophobic spheres. This implies that the retention of bacteriophages or other surrogates does not necessarily reflect the behavior of human pathogenic viruses because their hydrophobicity might be different. In this work, no clear differences were found between the three different viruses concerning their tendency to adsorb to drinking water biofilms in general, using qPCR. Although the target viruses differ in size, capsid structure, isoelectric point and type of genome, they accumulated in biofilms to a similar extent, using qPCR as detection method. On the contrary, increased accumulation of infectious HAdV in biofilms was found using cell culture, when incubated in hardened water (4 mM Ca^{2+}), whereas no such effect was observed using qPCR. This effect could be explained because HAdV has the lowest isoelectric point of the three viruses tested (Michen and Graule, 2010), thus the viral capsid would carry a negative surface charge at neutral pH (Gerba, 1984), which in turn would be expected to show the strongest attraction (among the three tested viruses) towards positive charges. On the other hand, these positive charges could be caused by accumulation of divalent cations such as Ca^{2+} in the EPS matrix of the biofilm. In a previous study, Michalowski (2012) found that the concentration of Ca^{2+} in 14-d-old drinking water biofilms was enriched by up to a factor of five compared to the bulk water. In addition to that, it is known that the concentration of divalent cations have an influence on the structure of the biofilm itself, because they increase the mechanical stability of biofilms (Korstgens et al., 2001; Shen et al., 2018) and influence the composition of the microbial community (Kelly et al., 2014). Although cell culture-based results from this work show that the retention of HAdV in drinking water biofilms might be enhanced by increased water hardness, the inconsistent results from qPCR and cell culture imply that further experiments are required to verify this hypothesis.

In this work, the persistence of viruses in biofilms over time periods longer than 7 d was not assessed, therefore it is not possible to estimate how long biofilms could retain viruses after the water is exchanged. Other studies showed that viruses including poliovirus, *Bacteroides fragilis* phage B40-8, coliphage MS2 and coliphage φX174 can be detected for a period of days up to several weeks when they are entrapped in the biofilm (Botzenhart and Hock, 2002; Storey and Ashbolt, 2001; Storey and Ashbolt, 2003a; Storey and Ashbolt, 2003b). Lehtola et al. (2007) found that the concentration of canine calicivirus RNA in biofilms was reduced by 30 % within three weeks, whereas the concentration in water decreased by 4 log units in the

same period of time, which clearly shows that aquatic biofilms can extend the duration of a contamination event. Similar conclusions were drawn by Helmi et al. (2008), who used rotating annular reactors with drinking water biofilms with a mixture of poliovirus, coliphage MS2 and coliphage φX174. After incorporation of the viruses into the biofilms, authors applied "draining operations" by exchanging the water of the system multiple times. While no poliovirus genomes were detected in water after the third draining operation, poliovirus was detected in the biofilm throughout the entire period of sampling (34 d), using qPCR (Helmi et al., 2008). The current literature about the interaction of viruses with aquatic biofilms is summarized in Table 14.

Table 14: Overview of literature about the interaction of viruses with aquatic biofilms.

Virus	Experimental setup	Detection method	Persistence of viruses	Reference
Bacteriophages (B40-8, MS2, ΦX174)	Three-month-old drinking water biofilms were grown on stainless steel coupons in modified robbins devices. Then, water with ~10^9 pfu/ml of each phage was recirculated in the system for 24 h, until the system was connected to tap water in flow through.	Plaque assay	Coliphage ΦX174 was detected in biofilms for a period of 20 d. Bacteriophages B40-8 and MS2 were detected in biofilms for the entire experimental period (30 d).	Storey and Ashbolt (2003a) Storey and Ashbolt (2003b)
Bacteriophages (B40-8, MS2)	72 d-old drinking water biofilms were grown on stainless steel and unplasticized polyvinyl chloride (uPVC) coupons in modified robbins devices. Coupons were transferred to annular reactors and incubated with ~10^8 pfu/ml of each phage under stagnant conditions.	Plaque assay	No phages were detected in biofilms grown on stainless steel coupons after 6 d of incubation. Bacteriophage B40-8 persisted in biofilms from uPVC coupons for the entire experimental period (30 d). Coliphage MS2 was detected for 22 d in biofilms from uPVC coupons.	Storey and Ashbolt (2001)
Canine calicivirus	One-month-old drinking water biofilms were grown on polyvinyl chloride (PVC) coupons in a propella biofilm reactor. Water with 4.5 x 10^6 gen.eq./ml canine calicivirus was flowing through the reactors for 2 h. Subsequently, reactors were supplied with drinking water without viruses for 4 weeks.	qPCR	Within 3 weeks, virus concentrations in biofilms decreased by 30 %, whereas concentrations in water decreased by ~4 log-units.	Lehtola et al. (2007)
Poliovirus type 1, Bacteriophages (MS2, ΦX174)	Seven-month-old drinking water biofilms and 14 d-old wastewater biofilms were grown on polycarbonate coupons in rotating annular reactors. All viruses were spiked to the reactors and incubated for 35 d. At different time points, draining operations were performed by exchanging the water in the reactor with drinking water without viruses.	Cell culture Plaque assay qPCR	Using cultural methods, all three viruses were detected in biofilms for a period of 6 d. Using qPCR, Polioviruses were detected in biofilms for the entire experimental period (34 d).	Helmi et al. (2008)
Poliovirus type 1	One-month-old drinking water biofilms were grown on polyvinyl chloride (PVC) in a pilot distribution system. Virus suspensions were pulse-injected for a period of 6 min into the distribution system.	Cell culture	Viruses accumulated in the biofilms compared to the bulk water by a factor of 2-10. Viruses were detected in the biofilm for the entire experimental period (24 h).	Quignon et al. (1997a, 1997b)

Table 14: Continued.

Virus	Experimental setup	Detection method	Persistence of viruses	Reference
Bacteriophages (MS2, GA, Qβ)	Two-month-old drinking water biofilms were grown on high density polyethylene (HDPE) coupons in rotating disc reactors. Bacteriophages were spiked to the reactors and incubated for a 1-10 h under stagnant conditions and 2-27 h under dynamic conditions.	qPCR	All phages were detected in biofilms during the entire period of sampling (10 h and 27 h, respectively).	Pelleieux et al. (2012)
Norovirus GGI, Bacteriophages (F-specific phages)	Two-month-old wastewater biofilms were grown on polyvinyl chloride (PVC) coupons in drinking water treatment plants and subsequently incubated in PBS for two months at 4 °C or 20 °C under stagnant conditions.	qPCR Plaque assay (only for bacteriophages)	All viruses were more persistent at 4°C than at 20°C, moreover all viruses were more persistent in biofilms than in corresponding water samples. Using qPCR, concentrations of norovirus GGI and F-specific phages in biofilms were not significantly reduced within 2 months. Using plaque assay, the concentration of F-specific phages in biofilms decreased by <1 log-unit within 2 months at 4 °C.	Skraber et al. (2009)
Bacteriophages (somatic coliphages, F-specific phages)	Two-month-old wastewater biofilms were grown on polyvinyl chloride (PVC) coupons in drinking water treatment plants and subsequently incubated in PBS for two months at 4 °C or 20 °C under stagnant conditions.	Plaque assay	For both groups of phages, a reduction <0.8 log-units/month was found for concentrations in biofilms at 4 °C and 20 °C. In water, the concentrations decreased by 2-10 log-units/month at 20 °C.	Skraber et al. (2007)
Poliovirus, Bacteriophages (MS2, ΦX174)	Two to nine weeks-old drinking water biofilms were grown under continuous flow conditions on glass slides covered with paraffin in biofilm reactors. Subsequently, viruses were added to the system and reactors were further incubated.	Cell culture, Plaque assay, qPCR (only for poliovirus)	Using cell culture, polioviruses were detected in biofilms for a period of 7 d. Using qPCR, polioviruses were detected in biofilms for a period of 14 d. Both coliphages were detected for 24 h after spiking, using plaque assay.	Botzenhart and Hock (2002)

Taken together, experiments showed that HAdV, MNV and coliphage φX174 accumulated in drinking water biofilms under both stagnant and flow conditions by up to 3.5 log units compared to the water phase, using culture independent quantification (qPCR). In addition to that, also infectious viruses were enriched in the biofilm, indicating that drinking water biofilms can be a reservoir for surrogates and human pathogenic viruses. The quantification of infectious MNV in biofilms using the eukaryotic cell line RAW 264.7 did not deliver plausible results, thus findings are still in question and require confirmation in the future. It is still to be proved that the accumulation is valid for other enteric viruses such as enteroviruses, rotaviruses or human noroviruses. Nevertheless, drinking water biofilms should be considered as potential reservoirs for enteric viruses after a fecal contamination of a drinking water distribution system had occurred, even when fecal indicators are no longer detected in water. The same was concluded by Miettinen et al. (2012), who studied a waterborne outbreak of gastroenteritis Nokia (Finland), caused by a fecal contamination of the drinking water distribution system. After the rectification of the contamination cause and immediate disinfection measures, HAdV were still detected in the drinking water for several months, whereas bacterial indicators were not detected.

6.2.2 Biological interactions of viruses in aquatic biofilms

Aquatic biofilms represent microbial ecosystems that harbor bacteria and protozoa which are embedded in a complex structure of EPS. Besides adsorption to components of the biofilm, also biological interactions might affect the fate of viruses within biofilms. For instance, protozoa could potentially ingest viruses and by that reduce or enhance virus persistence in the biofilm ecosystem. On the other hand, bacteriophages could infect their respective host bacteria in the biofilm, which could influence the composition and structure of the biofilm, but also the abundance of the phage itself. In this part of this work, two potential biological interactions were assessed in laboratory experiments. First, the uptake of HAdV by *A. castellanii* was elucidated under co-cultivation to determine the potential role of amoebae in virus retention. Second, the propagation of coliphage φX174 in *E. coli* monospecies biofilms was determined in microtiter plate assays under simulated environmental conditions. The latter was of particular interest because a propagation would compromise the indicator concept of coliphages in the aquatic environment.

6.2.2.1 *A. castellanii* as reservoir for HAdV

Amoebae are ubiquitous in aquatic systems, including surface waters, drinking water distribution systems and biofilms (Corsaro et al., 2010; Hoffmann and Michel, 2001). They graze on biofilms and take up biotic and abiotic particles, which can either be digested or excreted after a certain residence time. Studies showed that amoebae, including *Acanthamoeba*, can act as vectors for human pathogenic viruses (Lorenzo-Morales et al., 2007; Mattana et al., 2006; Scheid, 2015; Scheid and Schwarzenberger, 2012). Consequently, amoebae might play an important role in persistence and transmission of viruses in the urban water cycle. In a recent study, Atanasova et al. (2018) showed that internalized infectious coxsackieviruses B5 were detected in all life stages of *V. vermiformis* after co-cultivation, including the environmentally persistent cysts. The authors concluded that *V. vermiformis* might pose a new transmission pathway for viruses in water. Potentially, amoebae could also affect the fate of viruses in aquatic biofilms. However, no studies addressed this aspect until now.

In this work, *A. castellanii* was co-cultivated together with approximately 7.4 x 10^8 gen.eq./ml of HAdV in growth medium. Although the concentration of amoebae was not determined, it can be assumed that the viruses were present in excess. After one week of incubation at 25 °C, amoebae-associated HAdV were quantified using qPCR. HAdV were detected in *A. castellanii*, showing that the amoebae were capable to taking up HAdV during growth from the medium. Potentially, the results could be explained by attachment of HAdV to the cell surface of the amoebae, but this was assumed to be unlikely because several washing steps were applied to separate amoebae from viruses on the cell surface. With the experimental design used in this work, HAdV were not quantified using cultural methods, thus it is not possible to draw conclusions on viral infectivity. However, this work demonstrated that viruses were found inside the amoebae using culture-independent quantification (qPCR). Data from this work are supported by a study from Lorenzo-Morales et al. (2007), who isolated *Acanthamoeba* carrying adenovirus DNA from drinking water samples in Spain. Verani et al. (2016) found that *Acanthamoeba polyphaga* increases the tolerance of internalized adenoviruses towards chlorination, which clearly indicates the protective effect and thus hygienic relevance of amoebae in persistence of viruses in aquatic systems. Since amoebae are present in aquatic biofilms, they might also take up viruses that attached to the biofilm beforehand. To date, it is not known whether amoebae increase the persistence of viruses in aquatic biofilms. The overall aim of this study was to develop a flow cytometric assay to

quantify the uptake of HAdV by *A. castellanii* in drinking water biofilms. The flow cytometric assay was chosen because it allows the differentiation between amoebae, bacteria and other particles according to size and granularity, which would be mandatory to track viruses in the complex matrix of biofilms. In first experiments, HAdV were incubated with *A. castellanii* as described above and labelled with HAdV-specific antibodies prior measurement. Trying different protocols, it was not possible to detect either free HAdV or HAdV inside amoebae, using epifluorescence microscopy or flow cytometry, respectively (data not shown), indicating that most likely the antibody-labelling did not work. Interestingly, Verani et al. (2016) recently reported that the internalization of viruses by *Acanthamoeba polyphaga* is highly dependent on the presence of particulate matter in the growth medium. Similar to this work, the authors did not observe internalization of free viruses, using immunochemistry methods.

Within this study, it was only possible to show the uptake of HAdV by *A. castellanii* via qPCR in co-culture, but it remains unclear whether an uptake would also occur inside of drinking water biofilms. Future experiments are required to evaluate the relevance of the interaction between amoebae and viruses in aquatic biofilms.

6.2.2.2 Propagation of somatic coliphages in *E. coli* in monospecies biofilms

In many aspects, the behavior of coliphages in the environment is not comparable to that of human enteric viruses. While enteric viruses can't multiply in aquatic systems due to the absence of their respective host, coliphages could potentially propagate in the aquatic environment in presence of their natural bacterial host. On the other hand, also bacterial hosts have mechanisms to combat phage infections. In the presence of coliphages, phage resistant subpopulations of *E. coli* can develop within 24 h in a liquid culture, which were found to have a higher EPS production, potentially as a defense mechanism against bacteriophage attack (Lacqua et al., 2006). Refardt and Kümmerli (2013) found that an altruistic suicidal strain of *E. coli* which carrying the Rex abortive infection system successfully outcompeted a non-suicidal *E. coli* strain in the presence of coliphages in a structured environment. The authors concluded that besides phage resistance, bacterial altruism could also be a relevant defense mechanism against bacteriophages in structured environments.

While some studies reported a correlation between the occurrence of coliphages and enteric viruses (Haramoto et al., 2005; Jiang et al., 2001), other studies did not support such a

relationship (Hot et al., 2003; Jiang et al., 2007). There is a huge diversity of phages with different host spectra, but the detection via plaque assay focuses on two major groups: Somatic coliphages and F-specific coliphages. For both groups, specific host strains are established, although they might not be susceptible to infection by all coliphages that occur in the environment. Also vice versa, not all potential host strains that occur in the urban water cycle are necessarily infected by phages that were isolated using common host strains. For instance, Muniesa et al. (2003) used *E. coli* WG5 to isolate 25 different coliphages from the aquatic environment. Subsequently, the authors tested the host specificity of these phages and reported that only 3 % of isolated host bacteria, including clinical and environmental isolates from *E. coli*, *Shigella*, *Klebsiella* and *Leclercia* were sensitive to infection at least one of the phages. The complex interplay between coliphages and their naturally occurring hosts is not fully understood, however it might bias the results obtained from coliphage monitoring in urban water environments.

Besides water, also biofilms might be relevant in respect of phage/host interactions. The infection of *E. coli* inside of biofilms could potentially affect the structure and composition of the biofilm itself, moreover phages could propagate due to elevated concentrations of host cells compared to water. Infection of bacteria by phages always requires contact between phage and host, which is also determined by the abundance of both. Muniesa and Jofre (2004) reported that coliphage propagation requires concentrations of 10^4 *E. coli*/ml and 10^3 coliphages/ml, 10^3 *E. coli*/ml and 10^4 coliphages/ml, or intermediate values of both. The authors concluded that coliphage replication in water is unlikely because these concentrations are rarely found in natural water environments. However biofilms or sediments of surface waters might harbor elevated concentrations of both. In this work, bacteria and phages accumulated in surface water biofilms (0), which arises the question, whether a propagation of coliphages in biofilms would be feasible under environmental conditions. This would doubt the concept of coliphages as surrogates for enteric viruses in such aquatic environments.

In this work, the hypothesis was tested that infection of *E. coli* by somatic coliphages in biofilms primarily depends on growth temperature and nutrient conditions and thus is negligible in natural systems. To test this hypothesis, *E. coli* monospecies biofilms were grown in a microtiter plate format and subsequently exposed to different concentrations of somatic coliphage φX174 for a period of 4 h in growth medium or sterile filtered surface water from the river Ruhr in Essen (Germany) at different temperatures. The effect of coliphage φX174 on these biofilms was monitored by analyzing the phage titer in the

overlying medium by plaque assays at different time points and quantification of the remaining biofilms at the end of the incubation period. The latter showed that the biofilms were destroyed when the incubation was performed in growth medium at 36 °C, phage propagation was confirmed by an increase in virus titer over time. Potentially, an increase in phage concentration could also be explained by regrowth of planktonic bacteria from the biofilm and subsequent infection by phages, however the disruption of the biofilm indicates that also bacteria in the biofilm were infected and lysed. A higher initial phage concentration resulted in smaller biofilm residual after 4 h, probably due to a higher probability of infection in early stages of the incubation. Concerning biofilms incubated at 20 °C, the same trend was observed, however no increase in phage concentration was found after 4 h of incubation. This clearly shows that the propagation of coliphage φX174 in *E. coli* monospecies biofilms is temperature-dependent under favourable nutrient conditions. This finding was expected because the propagation of phages mainly depends on the growth rate of the host (Nabergoj et al., 2018; Woody and Cliver, 1995). To simulate natural conditions in surface waters of a temperate climate zone, incubation was carried out in sterile filtered surface water at 20 °C and, as a control, at 36 °C. No increase in phage concentration was observed within 4 h for both temperatures. Although an incubation of 4 h might have been too short to observe a significant propagation, it is likely that phage inactivation rates would be higher than propagation rates in a surface water environment. The fact that no phage propagation occurred within 4 h at the hosts optimal growth temperature in water indicates that the lack of nutrients and thus low metabolic activity of *E. coli* might hinder the reproduction of phages. In contrast to data from this study, Borrego et al. (1990) observed propagation of coliphage strains LL1.5, R3 and V2 in seawater at a temperature of 36 °C, however the concentration of organic matter might have been different and experiments in seawater might represent better physiological conditions compared to river water. Within this work, the quantification of residual biofilms after incubation in surface water was not a suitable tool to evaluate the effect of coliphage φX174 on *E. coli*, because the monospecies biofilms detached even in surface water without coliphages. Thus, no clear differences in residual biofilms were observed between the different phage concentrations.

To date, no study assessed the potential propagation of coliphages in aquatic biofilms of the urban environment. Providing preliminary data, this work demonstrated that the propagation of coliphage φX174 in *E. coli* monospecies biofilms is unlikely at typical water temperatures of a temperate climate zone. Even in tropical waters, a propagation would only be possible

under favourable nutrient conditions. Even if the latter might be found in naturally occurring multispecies biofilms, a significant propagation remains questionable, because concentrations of bacteria and phages would probably be lower than in this work, which further reduces the probability of infection (Wiggins and Alexander, 1985). In the past, some studies indicated that coliphages could propagate in the aquatic environment under certain conditions (Borrego et al., 1990; Seeley and Primrose, 1980; Vaughn and Metcalf, 1975), however other studies reported that a propagation can be assumed to be negligible or unlikely (Jofre et al., 2016; Muniesa et al., 2003; Woody and Cliver, 1995). Although biofilms represent an environment with increased microbial densities, this work showed that also the propagation of coliphages in surface water biofilms is negligible under natural conditions.

6.3 Conclusion and implications for future research

In this work, it was shown that aquatic biofilms in the urban water cycle can play an important role in retention of human and animal pathogenic viruses as well surrogate viruses. Biofilms are ubiquitous in all natural and technical systems and should be considered as potential reservoirs, not only for bacterial pathogens, but also relevant human viruses. In two field studies, this work showed that somatic coliphages, human- and animal pathogenic viruses tend to accumulate in surface water biofilms and -sediments, as well as biofilms from technical water systems in piglet breeding farms. Although a high prevalence of viruses was found in natural and technical systems of the urban water cycle, further research is required to elucidate the persistence and health relevance of viruses in aquatic biofilms. Besides field studies, this work revealed that enteric viruses and coliphages tend to accumulate in laboratory-scale drinking water biofilms by up to several orders of magnitude, which can have a high health significance in case of drinking water contamination events in practice. Not only viral DNA and RNA, but also infectious viruses were observed in elevated concentrations in the biofilm compared to the bulk water, regardless of flow conditions or water hardness. In model experiments, this work confirmed existing literature data that amoebae incorporate human enteric viruses in co-culture, however it remains unclear whether this phenomenon increases the persistence of viruses in aquatic biofilms. Further research is needed to investigate relevant biological interactions within the biofilm. Also interactions between somatic coliphages and their natural host *E. coli* in monospecies biofilms were investigated in this study, showing that propagation of phages under simulated environmental conditions of surface waters is unlikely.

7. References

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8. Appendix

8.1 qPCR amplicon sequences for customized DNA oligonucleotides

PAdV (based on Gen Bank Accession No. AJ237815.1)

AACGGCCGCTACTGCAAGTTCCACATCCAGGTGCCGCAAAAGTTCTTTGCCCTCA
AGAGCCTGCTGCT

HEV (based on Gen Bank Accession No. M73218.1)

GGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATCCCCTATATTCA
TCCAACCAACCCCT

8.2 Publikationsliste

Artikel in peer-reviewed journal

Leifels, M., Hamza, I. A., Wilhelm, M., Mackowiak, M., Jurzik, L., 2016. From lab to lake – Evaluation of current molecular methods for the detection of infectious enteric viruses in complex water matrices, PLoS One 11

Mackowiak, M., Leifels, M., Hamza, I.A., Jurzik, L., Wingender, J., 2018. Distribution of *Escherichia coli*, coliphages and enteric viruses in water, epilithic biofilms and sediments of an urban river in Germany. Sci Total Environ 626 650-659.

Konferenzbeitrag: Poster

Mackowiak, M., Leifels, M., Jurzik, L., Wingender, J.: Sediments and epilithic biofilms in surface waters are reservoirs for coliphages. VAAM-Jahrestagung, Jena, Deutschland, 13.-16.03.2016, ISSN: 0947-0867

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8.3 Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

8.4 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Interaction of enteric viruses with aquatic biofilms in the urban water cycle"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, 12.03.2019

Martin Mackowiak